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(54) Title: FLK-1 IS A RECEPTOR FOR VASCULAR ENDOTHELIAL GROWTH FACTOR

FLK-1 866 ILIHIGHHLNVNLLGACTKPGGPLMVIVEFSKFCNLSTYLRGKRNEFVPYKSKGARFRQ
KDR _____ C-D-S-T _____
TKR-C _____ C _____ S

FLK-1 926 GKDYVGELSVDLKRRLDSITSSQSSASSGFVEEKSLSDVEEEASEEELYKDFLTLEHLIC
KDR _____ AIP _____ P-D _____
TKR-C _____

FLK-1 986 YSFQVAKGMFLASRKC1HRDLAARNILLSEKNWKICDFGLARDIYKDPDYVRKGDLR
KDR _____
TKR-C _____

(57) Abstract

The present invention relates to the use of ligands for the Flk-1 receptor for the modulation of angiogenesis and vasculogenesis. The invention is based, in part, on the demonstration that Flk-1 tyrosine kinase receptor expression is associated with endothelial cells and the identification of vascular endothelial growth factor (VEGF) as the high affinity ligand of Flk-1. These results indicate a major role for Flk-1 in the signaling system during vasculogenesis and angiogenesis. Engineering of host cells that express Flk-1 and the uses of expressed Flk-1 to evaluate and screen for drugs and analogs of VEGF involved in Flk-1 modulation by either agonist or antagonist activities is described. The invention also relates to the use of FLK-1 ligands, including VEGF agonists and antagonists, in the treatment of disorders, including cancer, by modulating vasculogenesis and angiogenesis.

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Flk-1 IS A RECEPTOR FOR VASCULAR
ENDOTHELIAL GROWTH FACTOR

1. INTRODUCTION

5 The present invention relates to the use of ligands for the FLK-1 receptor for the modulation of angiogenesis and vasculogenesis. The invention is based, in part, on the demonstration that Flk-1 tyrosine kinase receptor expression is associated with endothelial cells and the 10 identification of vascular endothelial growth factor (VEGF) as the high affinity ligand of Flk-1. These results indicate a major role for Flk-1 in the signaling system during vasculogenesis and angiogenesis. Engineering of host cells that express Flk-1 and the uses 15 of expressed Flk-1 to evaluate and screen for drugs and analogs of VEGF involved in Flk-1 modulation by either agonist or antagonist activities is described.

20 The invention also relates to the use of FLK-1 ligands, including VEGF agonists and antagonists, in the treatment of disorders, including cancer, by modulating vasculogenesis and angiogenesis.

2. BACKGROUND OF THE INVENTION

25 Receptor tyrosine kinases comprise a large family of transmembrane receptors for polypeptide growth factors with diverse biological activities. Their intrinsic tyrosine kinase function is activated upon ligand binding, which results in phosphorylation of the receptor and multiple cellular substrates, and subsequently in a 30 variety of cellular responses (Ullrich A. and Schlessinger, J., 1990, Cell 61:203-212).

35 A receptor tyrosine kinase cDNA, designated fetal liver kinase 1 (Flk-1), was cloned from mouse cell populations enriched for hematopoietic stem and progenitor cells. The receptor was suggested to be involved in hematopoietic stem cell renewal (Matthews

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et al., 1991, Proc. Natl. Acad. Sci. USA 88:9026-9030). Sequence analysis of the Flk-1 clone revealed considerable homology with the c-Kit subfamily of receptor kinases and in particular to the Flt gene product. These receptors all have in common an extracellular domain containing immunoglobulin-like structures.

The formation and spreading of blood vessels, or vasculogenesis and angiogenesis, respectively, play important roles in a variety of physiological processes such as embryonic development, wound healing, organ regeneration and female reproductive processes such as follicle development in the corpus luteum during ovulation and placental growth after pregnancy. Uncontrolled angiogenesis can be pathological such as in the growth of solid tumors that rely on vascularization for growth.

Angiogenesis involves the proliferation, migration and infiltration of vascular endothelial cells, and is likely to be regulated by polypeptide growth factors. Several polypeptides with in vitro endothelial cell growth promoting activity have been identified. Examples include acidic and basic fibroblastic growth factor, vascular endothelial growth factor and placental growth factor. Although four distinct receptors for the different members of the FGF family have been characterized, none of these have as yet been reported to be expressed in blood vessels *in vivo*.

While the FGFs appear to be mitogens for a large number of different cell types, VEGF has recently been reported to be an endothelial cell specific mitogen (Ferrara, N. and Henzel, W.J., 1989, Biochem. Biophys. Res. Comm. 161:851-858). Recently, the fms-like tyrosine receptor, Flt, was shown to have affinity for VEGF (DeVries, C. et al., 1992, Science 255:989-991).

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3. SUMMARY OF THE INVENTION

The present invention relates to the use of ligands for the FLK-1 receptor for the modulation of angiogenesis and vasculogenesis. The present invention is based, in part, on the discovery that the Flk-1 tyrosine kinase receptor is expressed on the surface of endothelial cells and the identification of vascular endothelial growth factor (VEGF) as the high affinity ligand of Flk-1. The role of endothelial cell proliferation and migration during angiogenesis and vasculogenesis indicate an important role for Flk-1 in these processes. The invention is described by way of example for the murine Flk-1, however, the principles may be applied to other species including humans.

Pharmaceutical reagents designed to inhibit the Flk-1/VEGF interaction may be useful in inhibition of tumor growth. VEGF and/or VEGF agonists may be used to promote wound healing. The invention relates to expression systems designed to produce Flk-1 protein and/or cell lines which express the Flk-1 receptor. Expression of soluble recombinant Flk-1 protein may be used to screen peptide libraries for molecules that inhibit the Flk-1/VEGF interaction. Engineered cell lines expressing Flk-1 on their surface may be advantageously used to screen and identify VEGF agonists and antagonists.

4. BRIEF DESCRIPTION OF THE FIGURES

FIG. 1. Comparison of the Flk-1 amino acid sequence with related RTKs. Amino acid sequence comparison of Flk-1 with human KDR and rat TKr-C. A section of the sequence which is known for all three receptors is compared and only differences to the Flk-1 sequence are shown.

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FIG. 2. Northern blot analysis of Flk-1 gene expression. (A) Expression of Flk-1 RNA in day 9.5 to day 18.5 mouse embryos. Samples (10 μ g) of total RNA from whole mouse embryos were analyzed in each lane.

5 Positions of 28S and 18S ribosomal RNAs are marked.
(B) Expression of Flk-1 mRNA in postnatal day 4 and adult brain in comparison with capillary fragments from postnatal day 4 brain. 1 μ g of poly (A⁺) RNA was loaded on each lane. The 5' 2619 bp of the Flk-1 cDNA were used
10 as a probe. Control hybridization with a GAPDH cDNA probe is shown in the lower panel.

FIG. 3. Abundant Flk-1 gene expression in embryonic tissues. *In situ* hybridization analysis of Flk-1 expression in day 14.5 mouse embryo. (A) Bright
15 field illumination of a parasagittal section through the whole embryo hybridized with a ³⁵S-labeled antisense probe (5' 2619 bp). (B) Dark field illumination of the same section. (C) Control hybridization of an adjacent section with a sense probe. Abbreviations: Ao, aorta;
20 At, atrium; L, lung; Li, liver; Ma, mandible; Mn, meninges; Ms, mesencephalon; T, telencephalon; V, ventricle; Vt, vertebrae.

FIG. 4. Expression of Flk-1 RNA in embryonic organs is restricted to specific cells. Expression of Flk-1 RNA in a day 14.5 mouse embryo at higher magnification. (A) The heart region was probed with a ³⁵S-labeled antisense probe. (B) Adjacent section hybridized with the sense probe. (C) Part of the aorta wall shown on the cellular level. The endothelial cell-
30 layer is indicated by an arrow. (D) The lung, probed with the Flk-1 antisense probe. (E) Control hybridization of an adjacent section hybridized with the sense probe. Abbreviations: At, atrium; B, bronchus; Ed, endothelial cell layer; En, endocardium; L, lung, Li,

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liver; Lu, lumina of the aorta; M1, muscular; My, myocardium.

FIG. 5. Flk-1 gene expression in the brain of the developing mouse. In situ hybridization analysis of Flk-1 gene expression in the brain at different developmental stages. All sections were probed with the Flk-1 antisense probe. (A) Sagittal section of the telencephalon of a day 11.5 mouse embryo. A single blood vessel expressing Flk-1, which sprouts from the meninges into the neuroectoderm, is indicated by an arrow. (B) Sagittal sections of the brain of embryo day 14.5 and (C) of postnatal day 4. Shown are regions of the mesencephalon. Branching capillaries and blood vessels expressing Flk-1 are indicated by an arrow. (D) Sagittal section of an adult brain; a region of the mesencephalon is shown. Cells expressing Flk-1 are indicated by an arrow. Abbreviations: M, meninges; V, ventricle;

FIG. 6. Expression of Flk-1 in the choroid plexus of adult brain. (A) Darkfield illumination of the choroid plexus of an adult mouse brain hybridized with Flk-1 antisense probe. (B) Choroid plexus shown at a higher magnification. Arrows indicate single cells, which show strong expression of Flk-1. Abbreviations: CP, choroid plexus; E, ependyme; Ep, epithelial cells; V, ventricle.

FIG. 7. Flk-1 is expressed in the glomeruli of the kidney. (A) Parasagittal section of a 4-day postnatal kidney, hybridized with the Flk-1 antisense probe. (B) Hybridization signal accumulates in the glomeruli, as indicated by arrowheads. (C) Control hybridization of an adjacent section with the sense probe. (D) Sagittal section of an adult kidney probed with Flk-1. Arrowheads indicate glomeruli. (D) Glomerulus of an adult kidney at a higher magnification. The arrows in (A) and (D)

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indicate cells aligned in strands in the juxtaglomerular region expressing Flk-1.

FIG. 8. *In situ* hybridization analysis of Flk-1 expression in early embryos and extraembryonic tissues.

5 (A) Sagittal section of a day 8.5 mouse embryo in the maternal deciduum probed with Flk-1. (B) Higher magnification of the deciduum. Arrowheads indicate the endothelium of maternal blood vessels strongly expressing Flk-1 RNA. (C) High magnification of the yolk sac and
10 the trophectoderm of a day 9.5 mouse embryo. (D) High magnification of a blood island. Abbreviations:
A, allantois; Bi, blood island; Bv, maternal blood vessel; D, deciduum; En, endodermal layer of yolk sac; M, mesenchyme; Ms, mesodermal layer of yolk sac; NF, 15 neural fold; T, trophoblast; Y, yolk sac.

FIG. 9. Flk-1 is a receptor for VEGF. (A) Cross linking of ¹²⁵I-VEGF to COS cells transiently expressing the Flk-1 receptor and control cells were incubated with ¹²⁵I-VEGF at 4°C overnight, then washed twice with
20 phosphate buffered saline (PBS) and exposed to 0.5 mM of the cross linking agent DSS in PBS for 1 hour at 4°C. The cells were lysed, Flk-1 receptor immunoprecipitated, and analyzed by polyacrylamide gel electrophoresis followed by autoradiography. Molecular size markers are
25 indicated in kilodaltons. (B) Specific binding of ¹²⁵I-VEGF to COS cells expressing Flk-1. COS cells transiently expressing Flk-1 were removed from the plate and resuspended in binding medium (DMEM, 25 mM Hepes, 0.15% gelatin). Binding was performed at 15°C for 90
30 minutes in a total volume of 0.5 ml containing 2x10⁵ cells, 15,000 cpm ¹²⁵I-VEGF, and the indicated concentrations of unlabeled ligand. The cells were washed twice with PBS / 0.1% BSA and counted in a gamma counter.

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FIG. 10. VEGF-induced autophosphorylation of Flk-1. COS cells transiently expressing Flk-1 receptor and control cells were starved for 24 hours in DMEM containing 0.5% fetal calf serum and then stimulated with 5 VEGF for 10 minutes as indicated. The cells were solubilized, Flk-1 receptor immunoprecipitated with a polyclonal antibody against its C-terminus, separated by polyacrylamide gel electrophoresis, and transferred to nitrocellulose. The blot was probed with 10 antiphosphotyrosine antibodies (5B2). The protein bands were visualized by using a horseradish-peroxidase coupled secondary antibody and BCL™ (Amersham) detection assay.

FIG. 11. Nucleotide Sequence of Murine Flk-1.

FIG. 12. Plasmid Maps of retroviral vector

15 constructs. pLXSN Flk-1 TM Cl.1 and pLXSN Flk-1 TM cl.3 contain Flk-1 amino acids 1 through 806. pNTK-cfms-TM contains the 541 N-terminal amino acids of c-fms.

FIG. 13. Inhibition of C6 glioblastoma tumor growth by transdominant-negative inhibition of Flk-1. C6 cells 20 were implanted either alone or coimplanted with virus-producing cells. Cell numbers are as indicated in each panel. Two different virus-producing cell lines were used: one expressing the Flk-1 TM (transdominant-negative) mutant and one expressing a transdominant-negative c-fms mutant (c-fms TM) as a control. Beginning 25 at the time when the first tumors appeared, tumor volumes were measured every 2 to 3 days to obtain a growth curve. Each group is represented by four mice.

FIG. 14. Inhibition of C6 glioblastoma tumor growth 30 by transdominant-negative inhibition of Flk-1. C6 cells were implanted either alone or coimplanted with virus-producing cells. Cell numbers are as indicated in each panel. Two different virus-producing cell lines were used: one expressing the Flk-1 TM (transdominant-negative) mutant and one expressing a transdominant-

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negative c-fms mutant (cfms TM) as a control. Beginning at the time when the first tumor appeared, tumor volumes were measured every 2 to 3 days to obtain growth curve. Each group is represented by four mice.

5

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the use of ligands for the FLK-1 receptor to modulate angiogenesis and/or vasculogenesis. The invention also involves the 10 expression of Flk-1 to evaluate and screen for drugs and analogs of VEGF that may be involved in receptor activation, regulation and uncoupling. Such regulators of Flk-1 may be used therapeutically. For example, agonists of VEGF may be used in processes such as wound 15 healing; in contrast, antagonists of VEGF may be used in the treatment of tumors that rely on vascularization for growth.

The invention, is based, in part, on results from *in situ*-hybridization and Northern blot analyses indicating 20 that Flk-1 is an endothelial cell specific RTK. In addition, cross-linking experiments have shown Flk-1 to be a high affinity receptor for vascular endothelial growth factor (VEGF), indicating that Flk-1 plays a crucial role in the development and differentiation of 25 hemangioblast and in subsequent endothelial cell growth during vasculogenesis and angiogenesis.

The invention is based, also, on the discovery that expression of a transdominant-negative mutant form of the Flk-1 molecule can inhibit the biological activity of the 30 endogenous wild type Flk-1. Experiments are described herein, in which tumor cells and cells producing a recombinant retrovirus encoding a truncated Flk-1 receptor were mixed and injected into mice. Inhibition of vasculogenesis and growth of the injected tumor cells 35 was observed in mice expressing the truncated form of the

Flk-1 receptor. Expression of transdominant negative forms of the Flk-1 molecule may be useful for treatment of diseases resulting from abnormal proliferation of blood vessels, such as rheumatoid arthritis,
5 retinopathies and growth of solid tumors.

As explained in the working examples, infra, the polymerase chain reaction (PCR) method was used to isolate new receptor tyrosine kinases specifically expressed in post-implantation embryos and endothelial 10 cells. One such clone was found to encode a RTK that had almost identical sequence homology with the previously identified cDNA clone isolated from populations of cells enriched for hematopoietic cells and designated fetal liver kinase-1 (Flk-1) (Matthews et al., 1991, Proc. 15 Natl. Acad. Sci. U.S.A. 88:9026-9030) (FIG. 11).

For clarity of discussion, the invention is described in the subsections below by way of example for the murine Flk-1. However, the principles may be analogously applied to clone and express the Flk-1 of 20 other species including humans.

5.1. THE Flk-1 CODING SEQUENCE

The nucleotide coding sequence and deduced amino acid sequence of the murine Flk-1 gene is depicted in 25 Figure 11 (SEQ. ID NO. 1) and has recently been described in Matthews et al., 1991, Proc. Natl. Acad. Sci. U.S.A., 88:9026-9030. In accordance with the invention, the nucleotide sequence of the Flk-1 protein or its 30 functional equivalent in mammals, including humans, can be used to generate recombinant molecules which direct the expression of Flk-1; hereinafter, this receptor will be referred to as "Flk-1", regardless of the species from which it is derived.

In a specific embodiment described herein, the 35 murine Flk-1 gene was isolated by performing a polymerase

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chain reaction (PCR) using two degenerate oligonucleotide primer pools that were designed on the basis of highly conserved sequences within the kinase domain of receptor tyrosine kinases (Hanks et al., 1988,.) As a template, 5 DNA from a λgt10 cDNA library prepared from day 8.5 mouse embryos, was used. In a parallel approach, similar primers were used to amplify RTK cDNA sequences from capillary endothelial cells that had been isolated from the brains of post-natal day 4-8 mice. This is a time 10 when brain endothelial cell proliferation is maximal. Both approaches yielded cDNA sequences encoding the recently described fetal liver RTK, Flk-1 (Matthews et al., 1991). Based on amino acid homology, this receptor is a member of the type III subclass of RTKs (Ullrich and 15 Schlessinger) which contain immunoglobulin-like repeats in their extracellular domains (FIG. 1).

The invention also relates to Flk-1 genes isolated from other species, including humans, in which Flk-1 activity exists. Members of the Flk-1 family are defined 20 herein as those receptors that bind VEGF or fragments of the peptide. Such receptors may demonstrate about 80% homology at the amino acid level in substantial stretches of DNA sequence. A bacteriophage cDNA library may be screened, under conditions of reduced stringency, using a 25 radioactively labeled fragment of the mouse Flk-1 clone. Alternatively the mouse Flk-1 sequence can be used to design degenerate or fully degenerate oligonucleotide probes which can be used as PCR probes or to screen bacteriophage cDNA libraries. A polymerase chain 30 reaction (PCR) based strategy may be used to clone human Flk-1. Two pools of degenerate oligonucleotides, corresponding to a conserved motifs between the mouse Flk-1 and receptor tyrosine kinases, may be designed to serve as primers in a PCR reaction. The template for the 35 reaction is cDNA obtained by reverse transcription of

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mRNA prepared from cell lines or tissue known to express human Flk-1. The PCR product may be subcloned and sequenced to insure that the amplified sequences represent the Flk-1 sequences. The PCR fragment may be 5 used to isolate a full length Flk-1 cDNA clone by radioactively labeling the amplified fragment and screening a bacteriophage cDNA library. Alternatively, the labeled fragment may be used to screen a genomic library. For a review of cloning strategies which may be 10 used, see e.g., Maniatis, 1989, Molecular Cloning, A Laboratory Manual, Cold Springs Harbor Press, N.Y.; and Ausubel et al., 1989, Current Protocols in Molecular Biology, (Green Publishing Associates and Wiley Interscience, N.Y.)

15 Isolation of a human Flk-1 cDNA may also be achieved by construction of a cDNA library in a mammalian expression vector such as pcDNA1, that contains SV40 origin of replication sequences which permit high copy number expression of plasmids when transferred into COS 20 cells. The expression of Flk-1 on the surface of transfected COS cells may be detected in a number of ways, including the use of a labeled ligand such as VEGF or a VEGF agonist labeled with a radiolabel, fluorescent label or an enzyme. Cells expressing the human Flk-1 may 25 be enriched by subjecting transfected cells to a FACS (fluorescent activated cell sorter) sort.

In accordance with the invention, Flk-1 nucleotide sequences which encode Flk-1, peptide fragments of Flk-1, Flk-1 fusion proteins or functional equivalents thereof 30 may be used to generate recombinant DNA molecules that direct the expression of Flk-1 protein or a functionally equivalent thereof, in appropriate host cells. Alternatively, nucleotide sequences which hybridize to portions of the Flk-1 sequence may also be used in

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nucleic acid hybridization assays, Southern and Northern blot analyses, etc.

Due to the inherent degeneracy of the genetic code, other DNA sequences which encode substantially the same 5 or a functionally equivalent amino acid sequence, may be used in the practice of the invention for the cloning and expression of the Flk-1 protein. Such DNA sequences include those which are capable of hybridizing to the murine Flk-1 sequence under stringent conditions.

10 Altered DNA sequences which may be used in accordance with the invention include deletions, additions or substitutions of different nucleotide residues resulting in a sequence that encodes the same or a functionally equivalent gene product. The gene product itself may 15 contain deletions, additions or substitutions of amino acid residues within the Flk-1 sequence, which result in a silent change thus producing a functionally equivalent Flk-1. Such amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, 20 hydrophobicity, hydrophilicity, and/or the amphipatic nature of the residues involved. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; amino acids with uncharged polar head groups 25 having similar hydrophilicity values include the following: leucine, isoleucine, valine; glycine, analine; asparagine, glutamine; serine, threonine; phenylalanine, tyrosine. As used herein, a functionally equivalent Flk-1 refers to a receptor which binds to VEGF or fragments, 30 but not necessarily with the same binding affinity of its counterpart native Flk-1.

The DNA sequences of the invention may be engineered in order to alter the Flk-1 coding sequence for a variety of ends including but not limited to alterations which 35 modify processing and expression of the gene product.

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For example, mutations may be introduced using techniques which are well known in the art, e.g. site-directed mutagenesis, to insert new restriction sites, to alter glycosylation patterns, phosphorylation, etc. For 5 example, in certain expression systems such as yeast, host cells may over glycosylate the gene product. When using such expression systems it may be preferable to alter the Flk-1 coding sequence to eliminate any N-linked glycosylation site.

10 In another embodiment of the invention, the Flk-1 or a modified Flk-1 sequence may be ligated to a heterologous sequence to encode a fusion protein. For example, for screening of peptide libraries it may be useful to encode a chimeric Flk-1 protein expressing a 15 heterologous epitope that is recognized by a commercially available antibody. A fusion protein may also be engineered to contain a cleavage site located between the Flk-1 sequence and the heterologous protein sequence, so that the Flk-1 can be cleaved away from the heterologous 20 moiety.

In an alternate embodiment of the invention, the coding sequence of Flk-1 could be synthesized in whole or in part, using chemical methods well known in the art. See, for example, Caruthers, et al., 1980, Nuc. Acids 25 Res. Symp. Ser. 7:215-233; Crea and Horn, 1980, Nuc. Acids Res. 9(10):2331; Matteucci and Caruthers, 1980, Tetrahedron Letters 21:719; and Chow and Kempe, 1981, Nuc. Acids Res. 9(12):2807-2817. Alternatively, the protein itself could be produced using chemical methods 30 to synthesize the Flk-1 amino acid sequence in whole or in part. For example, peptides can be synthesized by solid phase techniques, cleaved from the resin, and purified by preparative high performance liquid chromatography. (E.g., see Creighton, 1983, Proteins 35 Structures And Molecular Principles, W.H. Freeman and

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Co., N.Y. pp. 50-60). The composition of the synthetic peptides may be confirmed by amino acid analysis or sequencing (e.g., the Edman degradation procedure; see Creighton, 1983, Proteins, Structures and Molecular 5 Principles, W.H. Freeman and Co., N.Y., pp. 34-49.

5.2. EXPRESSION OF Flk-1 RECEPTOR AND GENERATION OF CELL LINES THAT EXPRESS Flk-1

In order to express a biologically active Flk-1, the 10 nucleotide sequence coding for Flk-1, or a functional equivalent as described in Section 5.1 supra, is inserted into an appropriate expression vector, i.e., a vector which contains the necessary elements for the transcription and translation of the inserted coding 15 sequence. The Flk-1 gene products as well as host cells or cell lines transfected or transformed with recombinant Flk-1 expression vectors can be used for a variety of purposes. These include but are not limited to generating antibodies (i.e., monoclonal or polyclonal) 20 that bind to the receptor, including those that competitively inhibit binding of VEGF and "neutralize" activity of Flk-1 and the screening and selection of VEGF analogs or drugs that act via the Flk-1 receptor; etc.

25 5.2.1. EXPRESSION SYSTEMS

Methods which are well known to those skilled in the art can be used to construct expression vectors containing the Flk-1 coding sequence and appropriate transcriptional/translational control signals. These 30 methods include in vitro recombinant DNA techniques, synthetic techniques and in vivo recombination/genetic recombination. See, for example, the techniques described in Maniatis et al., 1989, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y. 35 and Ausubel et al., 1989, Current Protocols in Molecular

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Biology, Greene Publishing Associates and Wiley
Interscience, N.Y.

A variety of host-expression vector systems may be utilized to express the Flk-1 coding sequence. These 5 include but are not limited to microorganisms such as bacteria transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing the Flk-1 coding sequence; yeast transformed with recombinant yeast expression vectors containing the Flk-1 10 coding sequence; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing the Flk-1 coding sequence; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus; 15 TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing the Flk-1 coding sequence; or animal cell systems infected with recombinant virus expression vectors (e.g., adenovirus, vaccinia virus) including cell lines engineered to 20 contain multiple copies of the Flk-1 DNA either stably amplified (CHO/dhfr) or unstably amplified in double-minute chromosomes (e.g., murine cell lines).

The expression elements of these systems vary in their strength and specificities. Depending on the 25 host/vector system utilized, any of a number of suitable transcription and translation elements, including constitutive and inducible promoters, may be used in the expression vector. For example, when cloning in bacterial systems, inducible promoters such as pL of 30 bacteriophage λ , plac, ptrp, ptac (ptrp-lac hybrid promoter) and the like may be used; when cloning in insect cell systems, promoters such as the baculovirus polyhedrin promoter may be used; when cloning in plant cell systems, promoters derived from the genome of plant 35 cells (e.g., heat shock promoters; the promoter for the

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small subunit of RUBISCO; the promoter for the chlorophyll a/b binding protein) or from plant viruses (e.g., the 35S RNA promoter of CaMV; the coat protein promoter of TMV) may be used; when cloning in mammalian 5 cell systems, promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter) may be used; when generating cell lines that contain multiple copies of the 10 Flk-1 DNA SV40-, BPV- and EBV-based vectors may be used with an appropriate selectable marker.

In bacterial systems a number of expression vectors may be advantageously selected depending upon the use intended for the Flk-1 expressed. For example, when 15 large quantities of Flk-1 are to be produced for the generation of antibodies or to screen peptide libraries, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include but are not limited to 20 the E. coli expression vector pUR278 (Ruther et al., 1983, EMBO J. 2:1791), in which the Flk-1 coding sequence may be ligated into the vector in frame with the lac Z coding region so that a hybrid AS-lac Z protein is produced; pIN vectors (Inouye & Inouye, 1985, Nucleic 25 acids Res. 13:3101-3109; Van Heeke & Schuster, 1989, J. Biol. Chem. 264:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily 30 be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned polypeptide of interest 35 can be released from the GST moiety.

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In yeast, a number of vectors containing constitutive or inducible promoters may be used. For a review see, Current Protocols in Molecular Biology, Vol. 2, 1988, Ed. Ausubel et al., Greene Publish. Assoc. & Wiley Interscience, Ch. 13; Grant et al., 1987, Expression and Secretion Vectors for Yeast, in Methods in Enzymology, Eds. Wu & Grossman, 1987, Acad. Press, N.Y., Vol. 153, pp. 516-544; Glover, 1986, DNA Cloning, Vol. II, IRL Press, Wash., D.C., Ch. 3; and Bitter, 1987, Heterologous Gene Expression in Yeast, Methods in Enzymology, Eds. Berger & Kimmel, Acad. Press, N.Y., Vol. 152, pp. 673-684; and The Molecular Biology of the Yeast *Saccharomyces*, 1982, Eds. Strathern et al., Cold Spring Harbor Press, Vols. I and II.

In cases where plant expression vectors are used, the expression of the *Flk-1* coding sequence may be driven by any of a number of promoters. For example, viral promoters such as the 35S RNA and 19S RNA promoters of CaMV (Brisson et al., 1984, *Nature* 310:511-514), or the coat protein promoter of TMV (Takamatsu et al., 1987, *EMBO J.* 6:307-311) may be used; alternatively, plant promoters such as the small subunit of RUBISCO (Coruzzi et al., 1984, *EMBO J.* 3:1671-1680; Broglie et al., 1984, *Science* 224:838-843); or heat shock promoters, e.g., soybean hsp17.5-E or hsp17.3-B (Gurley et al., 1986, *Mol. Cell. Biol.* 6:559-565) may be used. These constructs can be introduced into plant cells using Ti plasmids, Ri plasmids, plant virus vectors, direct DNA transformation, microinjection, electroporation, etc. For reviews of such techniques see, for example, Weissbach & Weissbach, 1988, *Methods for Plant Molecular Biology*, Academic Press, NY, Section VIII, pp. 421-463; and Grierson & Corey, 1988, *Plant Molecular Biology*, 2d Ed., Blackie, London, Ch. 7-9.

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An alternative expression system which could be used to express Flk-1 is an insect system. In one such system, Autographa californica nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes.

5 The virus grows in Spodoptera frugiperda cells. The Flk-1 coding sequence may be cloned into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter). Successful insertion 10 of the Flk-1 coding sequence will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus (i.e., virus lacking the proteinaceous coat coded for by the polyhedrin gene). These recombinant viruses are then used to infect Spodoptera 15 frugiperda cells in which the inserted gene is expressed. (E.g., see Smith et al., 1983, J. Virol. 46:584; Smith, U.S. Patent No. 4,215,051).

In mammalian host cells, a number of viral based expression systems may be utilized. In cases where an 20 adenovirus is used as an expression vector, the Flk-1 coding sequence may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by in 25 vitro or in vivo recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing Flk-1 in infected hosts. (E.g., See Logan & Shenk, 1984, Proc. Natl. Acad. Sci. (USA) 81:3655-3659). Alternatively, the vaccinia 7.5K promoter 30 may be used. (See, e.g., Mackett et al., 1982, Proc. Natl. Acad. Sci. (USA) 79:7415-7419; Mackett et al., 1984, J. Virol. 49:857-864; Panicali et al., 1982, Proc. Natl. Acad. Sci. 79:4927-4931).

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Specific initiation signals may also be required for efficient translation of inserted Flk-1 coding sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where the entire Flk-1 gene, including its own initiation codon and adjacent sequences, is inserted into the appropriate expression vector, no additional translational control signals may be needed. However, in cases where only a portion of the Flk-1 coding sequence is inserted, exogenous translational control signals, including the ATG initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the Flk-1 coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (see Bittner et al., 1987, Methods in Enzymol. 153:516-544).

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such

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mammalian host cells include but are not limited to CHO, VERO, BHK, HeLa, COS, MDCK, 293, WI38, etc.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, 5 cell lines which stably express the Flk-1 may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with the Flk-1 DNA controlled by appropriate expression control elements (e.g., promoter, enhancer, 10 sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable 15 marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell 20 lines which express the Flk-1 on the cell surface, and which respond to VEGF mediated signal transduction. Such engineered cell lines are particularly useful in screening VEGF analogs.

A number of selection systems may be used, including 25 but not limited to the herpes simplex virus thymidine kinase (Wigler, et al., 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48:2026), and adenine phosphoribosyltransferase (Lowy, et al., 1980, Cell 30 22:817) genes can be employed in tk^r, hprt^r or aprt^r cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler, et al., 1980, Natl. Acad. Sci. USA 77:3567; O'Hare, et al., 1981, Proc. Natl. 35 Acad. Sci. USA 78:1527); gpt, which confers resistance to

mycophenolic acid (Mulligan & Berg, 1981), Proc. Natl. Acad. Sci. USA 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, et al., 1981, J. Mol. Biol. 150:1); and hygro, which confers resistance to hygromycin (Santerre, et al., 1984, Gene 30:147) genes. Recently, additional selectable genes have been described, namely trpB, which allows cells to utilize indole in place of tryptophan; hisD, which allows cells to utilize histinol in place of histidine (Hartman & Mulligan, 1988, Proc. Natl. Acad. Sci. USA 85:8047); and ODC (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue L., 1987, In: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory ed.).

5.2.2. IDENTIFICATION OF TRANSFECTANTS OR TRANSFORMANTS THAT EXPRESS THE Flk-1

The host cells which contain the coding sequence and which express the biologically active gene product may be identified by at least four general approaches; (a) DNA-DNA or DNA-RNA hybridization; (b) the presence or absence of "marker" gene functions; (c) assessing the level of transcription as measured by the expression of Flk-1 mRNA transcripts in the host cell; and (d) detection of the gene product as measured by immunoassay or by its biological activity.

In the first approach, the presence of the Flk-1 coding sequence inserted in the expression vector can be detected by DNA-DNA or DNA-RNA hybridization using probes comprising nucleotide sequences that are homologous to the Flk-1 coding sequence, respectively, or portions or derivatives thereof.

In the second approach, the recombinant expression vector/host system can be identified and selected based

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upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics, resistance to methotrexate, transformation phenotype, occlusion body formation in baculovirus, 5 etc.). For example, if the Flk-1 coding sequence is inserted within a marker gene sequence of the vector, recombinants containing the Flk-1 coding sequence can be identified by the absence of the marker gene function. Alternatively, a marker gene can be placed in tandem with 10 the Flk-1 sequence under the control of the same or different promoter used to control the expression of the Flk-1 coding sequence. Expression of the marker in response to induction or selection indicates expression of the Flk-1 coding sequence.

15 In the third approach, transcriptional activity for the Flk-1 coding region can be assessed by hybridization assays. For example, RNA can be isolated and analyzed by Northern blot using a probe homologous to the Flk-1 coding sequence or particular portions thereof.

20 Alternatively, total nucleic acids of the host cell may be extracted and assayed for hybridization to such probes.

25 In the fourth approach, the expression of the Flk-1 protein product can be assessed immunologically, for example by Western blots, immunoassays such as radioimmuno-precipitation, enzyme-linked immunoassays and the like. The ultimate test of the success of the expression system, however, involves the detection of the biologically active Flk-1 gene product. A number of 30 assays can be used to detect receptor activity including but not limited to VEGF binding assays; and VEGF biological assays using engineered cell lines as the test substrate.

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5.3. USES OF THE Flk-1 RECEPTOR
AND ENGINEERED CELL LINES

Angiogenesis, the growth of new blood capillary vessels, is required for a number of physiological processes ranging from wound healing, tissue and organ regeneration, placental formation after pregnancy and embryonic development. Abnormal proliferation of blood vessels is an important component of a variety of diseases such as rheumatoid arthritis, retinopathies, and psoriasis. Angiogenesis is also an important factor in the growth and metastatic activity of solid tumors that rely on vascularization. Therefore, inhibitors of angiogenesis may be used therapeutically for the treatment of diseases resulting from or accompanied by abnormal growth of blood vessels and for treatments of malignancies involving growth and spread of solid tumors.

In an embodiment of the invention the Flk-1 receptor and/or cell lines that express the Flk-1 receptor may be used to screen for antibodies, peptides, or other ligands that act as agonists or antagonists of angiogenesis or vasculogenesis mediated by the Flk-1 receptor. For example, anti-Flk-1 antibodies capable of neutralizing the activity of VEGF, may be used to inhibit Flk-1 function. Additionally, anti-Flk-1 antibodies which mimic VEGF activity may be selected for uses in wound healing. Alternatively, screening of peptide libraries with recombinantly expressed soluble Flk-1 protein or cell lines expressing Flk-1 protein may be useful for identification of therapeutic molecules that function by inhibiting the biological activity of Flk-1.

In an embodiment of the invention, engineered cell lines which express the entire Flk-1 coding region or its ligand binding domain may be utilized to screen and identify VEGF antagonists as well as agonists. Synthetic compounds, natural products, and other sources of

potentially biologically active materials can be screened in a number of ways. The ability of a test compound to inhibit binding of VEGF to Flk-1 may be measured using standard receptor binding techniques, such as those

5 described in Section 6.1.9. The ability of agents to prevent or mimic, the effect of VEGF binding on signal transduction responses on Flk-1 expressing cells may be measured. For example, responses such as activation of Flk-1 kinase activity, modulation of second messenger
10 production or changes in cellular metabolism may be monitored. These assays may be performed using conventional techniques developed for these purposes.

15 5.3.1. SCREENING OF PEPTIDE LIBRARY WITH
Flk-1 PROTEIN OR ENGINEERED CELL LINES

Random peptide libraries consisting of all possible combinations of amino acids attached to a solid phase support may be used to identify peptides that are able to bind to the ligand binding site of a given receptor or
20 other functional domains of a receptor such as kinase domains (Lam, K.S. et al., 1991, Nature 354: 82-84). The screening of peptide libraries may have therapeutic value in the discovery of pharmaceutical agents that act to inhibit the biological activity of receptors through
25 their interactions with the given receptor.

Identification of molecules that are able to bind to the Flk-1 may be accomplished by screening a peptide library with recombinant soluble Flk-1 protein. Methods for expression and purification of Flk-1 are described in
30 Section 5.2.1 and may be used to express recombinant full length Flk-1 or fragments of Flk-1 depending on the functional domains of interest. For example, the kinase and extracellular ligand binding domains of Flk-1 may be separately expressed and used to screen peptide
35 libraries.

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To identify and isolate the peptide/solid phase support that interacts and forms a complex with Flk-1, it is necessary to label or "tag" the Flk-1 molecule. The Flk-1 protein may be conjugated to enzymes such as

5 alkaline phosphatase or horseradish peroxidase or to other reagents such as fluorescent labels which may include fluorescein isothiocyanate (FITC), phycoerythrin (PE) or rhodamine. Conjugation of any given label, to Flk-1, may be performed using techniques that are routine

10 in the art. Alternatively, Flk-1 expression vectors may be engineered to express a chimeric Flk-1 protein containing an epitope for which a commercially available antibody exist. The epitope specific antibody may be tagged using methods well known in the art including

15 labeling with enzymes, fluorescent dyes or colored or magnetic beads.

The "tagged" Flk-1 conjugate is incubated with the random peptide library for 30 minutes to one hour at 22°C to allow complex formation between Flk-1 and peptide species within the library. The library is then washed to remove any unbound Flk-1 protein. If Flk-1 has been conjugated to alkaline phosphatase or horseradish peroxidase the whole library is poured into a petri dish containing a substrates for either alkaline phosphatase or peroxidase, for example, 5-bromo-4-chloro-3-indoyl phosphate (BCIP) or 3,3',4,4"-diaminobenzidine (DAB), respectively. After incubating for several minutes, the peptide/solid phase-Flk-1 complex changes color, and can be easily identified and isolated physically under a dissecting microscope with a micromanipulator. If a fluorescent tagged Flk-1 molecule has been used, complexes may be isolated by fluorescent activated sorting. If a chimeric Flk-1 protein expressing a heterologous epitope has been used, detection of the peptide/Flk-1 complex may be accomplished by using a

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labeled epitope specific antibody. Once isolated, the identity of the peptide attached to the solid phase support may be determined by peptide sequencing.

In addition to using soluble Flk-1 molecules, in 5 another embodiment, it is possible to detect peptides that bind to cell surface receptors using intact cells. The use of intact cells is preferred for use with receptors that are multi-subunits or labile or with receptors that require the lipid domain of the cell 10 membrane to be functional. Methods for generating cell lines expressing Flk-1 are described in Sections 5.2.1. and 5.2.2. The cells used in this technique may be either live or fixed cells. The cells will be incubated with the random peptide library and will bind to certain 15 peptides in the library to form a "rosette" between the target cells and the relevant solid phase support/peptide. The rosette can thereafter be isolated by differential centrifugation or removed physically under a dissecting microscope.

20 As an alternative to whole cell assays for membrane bound receptors or receptors that require the lipid domain of the cell membrane to be functional, the receptor molecules can be reconstituted into liposomes where label or "tag" can be attached.

25

5.3.2. ANTIBODY PRODUCTION AND SCREENING

Various procedures known in the art may be used for the production of antibodies to epitopes of the 30 recombinantly produced Flk-1 receptor. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments and fragments produced by an Fab expression library. Neutralizing antibodies i.e., those which compete for the VEGF binding 35

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site of the receptor are especially preferred for diagnostics and therapeutics.

Monoclonal antibodies that bind Flk-1 may be radioactively labeled allowing one to follow their

- 5 location and distribution in the body after injection. Radioactivity tagged antibodies may be used as a non-invasive diagnostic tool for imaging *de novo* vascularization associated with a number of diseases including rheumatoid arthritis, macular degeneration, and
- 10 formation of tumors and metastases.

Immuno-toxins may also be designed which target cytotoxic agents to specific sites in the body. For example, high affinity Flk-1 specific monoclonal antibodies may be covalently complexed to bacterial or

- 15 plant toxins, such as diphteria toxin, abrin or ricin. A general method of preparation of antibody/hybrid molecules may involve use of thiol-crosslinking reagents such as SPDP, which attack the primary amino groups on the antibody and by disulfide exchange, attach the toxin
- 20 to the antibody. The hybrid antibodies may be used to specifically eliminate Flk-1 expressing endothelial cells.

For the production of antibodies, various host animals may be immunized by injection with the Flk-1

- 25 protein including but not limited to rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide,
- 30 surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and Corynebacterium parvum.

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Monoclonal antibodies to Flk-1 may be prepared by using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include but are not limited to the hybridoma

- 5 technique originally described by Kohler and Milstein, (Nature, 1975, 256:495-497), the human B-cell hybridoma technique (Kosbor et al., 1983, Immunology Today, 4:72; Cote et al., 1983, Proc. Natl. Acad. Sci., 80:2026-2030) and the EBV-hybridoma technique (Cole et al., 1985,
- 10 Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). In addition, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci., 81:6851-6855; Neuberger et al., 1984, Nature, 312:604-608; Takeda et al., 1985,
- 15 Nature, 314:452-454) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used.

Alternatively, techniques described for the production of

- 20 single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce Flk-1-specific single chain antibodies.

Antibody fragments which contain specific binding sites of Flk-1 may be generated by known techniques. For example, such fragments include but are not limited to: the F(ab'), fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the F(ab'), fragments. Alternatively, Fab expression libraries may be constructed (Huse et al., 1989, Science, 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity to Flk-1.

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The Flk-1 coding sequence may be used for diagnostic purposes for detection of Flk-1 expression. Included in the scope of the invention are oligoribonucleotide sequences, that include antisense RNA and DNA molecules and ribozymes that function to inhibit translation of Flk-1. In addition, mutated forms of Flk-1, having a dominant negative effect, may be expressed in targeted cell populations to inhibit the activity of endogenously expressed wild-type Flk-1.

10

5.4.1. USE OF Flk-1 CODING SEQUENCE
IN DIAGNOSTICS AND THERAPEUTICS

The Flk-1 DNA may have a number of uses for the diagnosis of diseases resulting from aberrant expression 15 of Flk-1. For example, the Flk-1 DNA sequence may be used in hybridization assays of biopsies or autopsies to diagnose abnormalities of Flk-1 expression; e.g., Southern or Northern analysis, including in situ hybridization assays.

20 The Flk-1 cDNA may be used as a probe to detect the expression of the Flk-1 mRNA. In a specific example described herein, the expression of Flk-1 mRNA in mouse embryos of different developmental stages was analyzed. Northern blot analysis indicated abundant expression of a 25 major 5.5 kb mRNA between day 9.5 and day 18.5, with apparent decline towards the end of gestation (FIG. 2A). In post-natal day 4-8 brain capillaries Flk-1 mRNA was found to be highly enriched compared to total brain RNA (FIG. 2B), suggesting a role for Flk-1 in endothelial cell 30 proliferation.

To obtain more detailed information about the expression of Flk-1 during embryonic development and during the early stages of vascular development in situ hybridization experiments were performed as described in 35 Section 6.1.4. In situ hybridizations demonstrated that

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Flk-1 expression *in vivo* during embryonic mouse development is largely restricted to endothelial cells and their precursors (FIG. 3 and FIG. 4). Flk-1 is expressed in endothelial cells during physiological processes that are characterized by endothelial cell proliferation and the temporal and spatial expression pattern found in the embryonic brain correlate precisely with the development of the neural vascular system as described by Bar (1980). Vascular sprouts originating in the perineural plexus grow radially into the neuroectoderm and branch there and these sprouts were found to express high amounts of Flk-1 mRNA (FIG. 5). In the early postnatal stages endothelial cell proliferation is still evident and Flk-1 is expressed, whereas in the adult organism, after completion of the vascularization process, the decline in endothelial cell proliferation parallels a decrease in Flk-1 expression.

Also within the scope of the invention are oligoribonucleotide sequences, that include anti-sense RNA and DNA molecules and ribozymes that function to inhibit the translation of Flk-1 mRNA. Anti-sense RNA and DNA molecules act to directly block the translation of mRNA by binding to targeted mRNA and preventing protein translation. In regard to antisense DNA, oligodeoxyribonucleotides derived from the translation initiation site, e.g., between -10 and +10 regions of the Flk-1 nucleotide sequence, are preferred.

Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by a endonucleolytic cleavage. Within the scope of the invention are engineered hammerhead motif ribozyme molecules that specifically and

35

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efficiently catalyze endonucleolytic cleavage of Flk-1 RNA sequences.

Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites which include the following sequences, GUA, GUU and GUC. Once identified, short RNA sequences of between 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site may be evaluated for predicted structural features such as secondary structure that may render the oligonucleotide sequence unsuitable. The suitability of candidate targets may also be evaluated by testing their accessibility to hybridization with complementary oligonucleotides, using ribonuclease protection assays.

Both anti-sense RNA and DNA molecules and ribozymes of the invention may be prepared by any method known in the art for the synthesis of RNA molecules. These include techniques for chemically synthesizing oligodeoxyribonucleotides well known in the art such as for example solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by *in vitro* and *in vivo* transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors which incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.

Various modifications to the DNA molecules may be introduced as a means of increasing intracellular stability and half-life. Possible modifications include but are not limited to the addition of flanking sequences of ribo- or deoxy- nucleotides to the 5' and/or 3' ends

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of the molecule or the use of phosphorothioate or 2' O-methyl rather than phosphodiesterase linkages within the oligodeoxyribonucleotide backbone.

5 5.4.2. USE OF DOMINANT NEGATIVE
Flk-1 MUTANTS IN GENE THERAPY

Receptor dimerization induced by ligands, is thought to provide an allosteric regulatory signal that functions to couple ligand binding to stimulation of kinase 10 activity. Defective receptors can function as dominant negative mutations by suppressing the activation and response of normal receptors by formation of unproductive heterodimers. Therefore, defective receptors can be engineered into recombinant viral vectors and used in 15 gene therapy in individuals that inappropriately express Flk-1.

In an embodiment of the invention, mutant forms of the Flk-1 molecule having a dominant negative effect may be identified by expression in selected cells. Deletion 20 or missense mutants of Flk-1 that retain the ability to form dimers with wild type Flk-1 protein but cannot function in signal transduction may be used to inhibit the biological activity of the endogenous wild type Flk-1. For example, the cytoplasmic kinase domain of Flk-1 25 may be deleted resulting in a truncated Flk-1 molecule that is still able to undergo dimerization with endogenous wild type receptors but unable to transduce a signal.

Abnormal proliferation of blood vessels is an 30 important component of a variety of pathogenic disorders such as rheumatoid arthritis, retinopathies and psoriasis. Uncontrolled angiogenesis is also an important factor in the growth and metastases of solid tumors. Recombinant viruses may be engineered to express 35 dominant negative forms of Flk-1 which may be used to

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inhibit the activity of the wild type endogenous Flk-1. These viruses may be used therapeutically for treatment of diseases resulting from aberrant expression or activity of Flk-1.

5 Expression vectors derived from viruses such as retroviruses, vaccinia virus, adeno-associated virus, herpes viruses, or bovine papilloma virus, may be used for delivery of recombinant Flk-1 into the targeted cell population. Methods which are well known to those skilled in the art can be used to construct recombinant viral vectors containing Flk-1 coding sequence. See, for example, the techniques described in Maniatis et al., 10 1989, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y. and Ausubel et al., 1989, Current 15 Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, N.Y. Alternatively, recombinant Flk-1 molecules can be reconstituted into liposomes for delivery to target cells.

In a specific embodiment of the invention, a 20 deletion mutant of the Flk-1 receptor was engineered into a recombinant retroviral vector. Two clonal isolates designated pLXSN Flk-1 TM cl.1 and pLXSN Flk-1 TM cl.3 contain a truncated Flk-1 receptor lacking the 561 COOH-terminal amino acids. To obtain virus producing cell 25 lines, PA37 cells were transfected with the recombinant vectors and, subsequently, conditioned media containing virus were used to infect GPE cells.

To test whether expression of signaling-defective mutants inhibits endogenous Flk-1 receptor activity, C6 30 rat glioblastoma cells (tumor cells) and mouse cells producing the recombinant retroviruses were mixed and injected subcutaneously into nude mice. Normally, injection of tumor cells into nude mice would result in proliferation of the tumor cells and vascularization of 35 the resulting tumor mass. Since Flk-1 is believed to be

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essential for formation of blood vessels, blocking Flk-1 activity by expression of a truncated receptor, might function to inhibit vascularization of the developing tumor and, thereby, inhibit its growth. As illustrated 5 in Figures 13 and 14, coinjection of virus producing cells, expressing a truncated Flk-1 receptor, significantly inhibits the growth of the tumor as compared to controls receiving only tumor cells.

10 5.5. USE OF Flk-1 RECEPTOR OR LIGANDS

Receptor/ligand interaction between Flk-1 and VEGF is believed to play an important role in the signalling system during vascularization and angiogenesis. Abnormal proliferation of blood vessels is an important component 15 of a number of diseases.

Expression of Flk-1 RNA correlates with the development of the brain and with endothelial cell proliferation suggesting that Flk-1 might be a receptor involved in mediation of signaling events in the 20 vascularization process. VEGF has been shown to be a mitogenic growth factor known to act exclusively on endothelial cell (Ferrara, N. and Henzel, W.J., 1989, Biochem. Biophys. Res. Comm. 161:851-858). Cross-linking and ligand binding experiments were performed, as 25 described in Section 6.1.9 and 6.1.10 respectively, to determine whether VEGF is a ligand for Flk-1 and the results indicate that Flk-1 is an authentic high affinity VEGF receptor (FIG 9).

In one embodiment of the invention, ligands for 30 Flk-1, the Flk-1 receptor itself, or a fragment containing its VEGF binding site, could be administered in vivo to modulate angiogenesis and/or vasculogenesis. For example, administration of the Flk-1 receptor or a fragment containing the VEGF binding site, could 35 competitively bind to VEGF and inhibit its interaction

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with the native Flk-1 receptor *in vivo* to inhibit angiogenesis and/or vasculogenesis. Alternatively, ligands for Flk-1, including anti-Flk-1 antibodies or fragments thereof, may be used to modulate angiogenesis 5 and/or vasculogenesis. Agonists of VEGF activity may be used to promote wound healing whereas antagonists of VEGF activity may be used to inhibit tumor growth.

Depending on the specific conditions being treated, these agents may be formulated and administered 10 systemically or locally. Techniques for formulation and administration may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition. Suitable routes may include oral, rectal, transmucosal, or intestinal administration; 15 parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections, just to name a few. For injection, the agents of the 20 invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. For such transmucosal administration, penetrants appropriate to the barrier to be permeated are 25 used in the formulation. Such penetrants are generally known in the art.

6. EXAMPLE: CLONING AND EXPRESSION PATTERNS
OF Flk-1, A HIGH AFFINITY
RECEPTOR FOR VEGF

30 The subsection below describes the cloning and characterization of the Flk-1 cDNA clone. Northern blot and *in situ* hybridization analyses indicate that Flk-1 is expressed in endothelial cells. Cross-linking and ligand binding experiments further indicate that Flk-1 is a high 35 affinity receptor for VEGF.

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6.1. MATERIALS AND METHODS

6.1.1. cDNA CLONING OF Flk-1

DNA extracted from λgt10 cDNA library of day 8.5
5 mouse embryos (Fahrner et al., 1987, EMBO. J. 6:1497-
1508) was used as template for polymerase chain reaction
(PCR; Saiki, R.K. et al., 1985 Science 230:1350-1354).
In an independent approach cDNA of capillary endothelial
cells that had been isolated from the brain of postnatal
10 day 4-8 mice was used for amplification (Risau, W., 1990
In: development of the Vascular System. Issues Biomed.
Basel Karger 58-68 and Schnürch et al., unpublished)
Degenerated primers were designed on the basis of high
amino acid homologies within the kinase domain shared by
15 all RTKs (Wilks, A.F., 1989, Proc. Natl. Acad. Sci.
U.S.A. 86:1603-1607).

Full length cDNA clones of Flk-1 were isolated from
another day 8.5 mouse embryo cDNA library, which had been
prepared according to the method of Okayama and Berg
20 (1983), and a day 11.5 mouse embryo λgt11 library
(Clonetech) using the ³²P-labeled (Feinberg, A.P. and
Vogelstein, B. 1983 Anal. Biochem. 132:6-13) 210-bp PCR
fragment.

25 6.1.2. MOUSE EMBRYOS

Balb/c mice were mated overnight and the morning of
vaginal plug detection was defined as 1/2 day of
gestation. For Northern blot analysis the frozen embryos
were homogenized in 5 M guanidinium thiocyanate and RNA
30 was isolated as described (Ullrich, A. et al., 1985,
Nature 313:756-761). For in situ hybridization, the
embryos were embedded in Tissue-Tek (Miles), frozen on
the surface of liquid nitrogen and stored at -70C prior
to use.

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6.1.3. PREPARATION OF PROBES

The 5'-located 2619 bp of the receptor cDNA were subcloned in the pGem3Z vector (Promega) as an EcoR1/BamH1 fragment. The probe for Northern blot hybridization was prepared by labelling the cDNA fragment with α -³²PdATP (Amersham) by random hexanucleotide priming (Boehringer; Feinberg, A.P. and Vogelstein, B., 1983 Anal. Biochem. 132:6-13).

For *in situ* hybridization a single-strand antisense DNA probe was prepared as described by Schnürch and Risau (Development, 1991 111:1143-54). The plasmid was linearized at the 3' end of the cDNA and a sense transcript was synthesized using SP6 RNA polymerase (Boehringer). The DNA was degraded using DNAase (RNAase free preparation, Boehringer Mannheim). With the transcript, a random-primed cDNA synthesis with a α -³⁵S dATP (Amersham) was performed by reverse transcription with MMLV reverse transcriptase (BRL). To obtain small cDNA fragments of about 100 bp in average suitable for *in situ* hybridization, a high excess of primer was used. Subsequently the RNA transcript was partially hydrolyzed in 100 mM NaOH for 20 minutes at 70°C, and the probe was neutralized with the same amount of HCl and purified with a Sephadex C50 column. After ethanol precipitation the probe was dissolved at a final specific activity of 5×10^5 cpm. For control hybridization a sense probe was prepared with the same method.

6.1.4. RNA EXTRACTION AND NORTHERN ANALYSIS

Total cytoplasmic RNA was isolated according to the acidic phenol-method of Chromczynski and Sacchi (1987). Poly(A⁺) RNA aliquots were electrophoresed in 1.2% agarose formaldehyde (Sambrook, J. et al., 1989 Molecular Cloning: A Laboratory Manual 2nd ed. Cold Spring Harbor Laboratory Press) gels and transferred to nitrocellulose

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membranes (Schleicher & Schuell), Hybridizations were performed overnight in 50% formamide, 5 x SSC (750mM sodium chloride, 75mM sodium citrate), 5 x Denhardt's (0.1% Ficoll 400, 0.1% polyvinylpryollidone, 0.1% BSA)

5 and -0.5% SDS at 42°C with 1-3x10⁶ cpm-ml⁻¹ of ³²P-Random primed DNA probe, followed by high stringency washes in 0.2 x SSC, 0.5% SDS at 52°C. The filters were exposed for 4 to 8 days.

10 6.1.5. IN SITU HYBRIDIZATION

Subcloning postfixation and hybridization was essentially performed according to Hogan et al. (1986). 10 µm thick sections were cut at -18°C on a Leitz cryostat. For prehybridization treatment no incubation

15 with 0.2M HCl for removing the basic proteins was performed. Sections were incubated with the ³⁵S-cDNA probe (5x10⁴cpm/µl) at 52°C in a buffer containing 50% formamide, 300 mM NaCl, 10 mM Tris-HCl, 10 mM NaPO₄ (pH 6.8), 5 mM EDTA, 0.02% Ficoll 400, 0.01%

20 polyvinylprolidone 0.02% BSA 10 m /ml yeast RNA, 10% dextran sulfate, and 10 mM NaCl, 10 mM Tris-HCl, 10 mM NaPO₄ (pH 6.8), 5 mM EDTA, 10 Mm DTT at 52°C). For autoradiography, slides were coated with Kodak NTB2 film emulsion and exposed for eight days. After developing,

25 the sections were counterstained and toluidine blue or May-Grinwald.

6.1.6. PREPARATION OF ANTISERA

The 3' primed EcoRV/HindII fragment comprising the 30 128 C-terminal amino acids of Flk-1 was subcloned in the fusion protein expression vector pGEX3X (Smith, D.B. and Johnson, K.S., 1990 Gene. 67:31-40; Pharmacia). The fusion protein was purified as described and used for immunizing rabbits. After the second boost the rabbits

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were bled and the antiserum was used for immunoprecipitation.

6.1.7. TRANSIENT EXPRESSION
5 OF Flk-1 IN COS-1 CELLS

Transfection of COS-1 cells was performed essentially as described by Chen and Okayama (1987 Mol. Cell. Biol. 7:2745-2752) and Gorman et al. (1989 Virology 171:377-385). Briefly, cells were seeded to a density of 10 1.0 x 10⁶ per 10-cm dish and incubated overnight in DMEM containing 10% fetal calf serum (Gibco). 20 µg of receptor cDNA cloned into a cytomegalovirus promotor driven expression vector was mixed in 0.5 ml of 0.25 M CaCl₂, 0.5 ml of 2 x BBS (280 mM NaCl, 1.5 mM Na₂HPO₄, 50 15 mM BES, pH 6.96 and incubated for 30 min at room temperature. The calcium phosphate/DNA solution was then added to the cells, swirled gently, and incubated for 18 hours at 37°C under 3% CO₂. For ligand binding experiments, the cells were removed from the plate and 20 treated as described below.

To obtain VEGF conditioned media, cells were transfected in 15-cm dishes. Media was collected after 48 h and VEGF was partially purified by affinity chromatography using heparin High Trap TM columns 25 (Pharmacia) and concentrated by ultrafiltration (Ferrara, N. and Henzel, W.J. 1989 Biochem. Biophys. Res. Comm. 161:851-858). The concentration of VEGF was determined by a ligand competition assay with bovine aortic endothelial cells.

30 For autophosphorylation assays, cells were seeded in 6-well dishes (2x10⁵ cells per well), transfected as described above, and starved for 24 h in DMEM containing 0.5% fetal calf serum. The cells were then treated with 500 pM VEGF for 10 min. at 37°C or left untreated and 35 were subsequently lysed as described by Kris et al.

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(1985). Flk-1 was immunoprecipitated with an antiserum raised in rabbits against the C-terminus of the receptor. The immunoprecipitates were separated on a 7.5% SDS polyacrylamide gel, transferred to nitrocellulose, and 5 incubated with a mouse monoclonal antibody directed against phosphotyrosine (5E2; Fendly, B.M. et al., 1990 Cancer Research 50:1550-1558). Protein bands were visualized using horseradish peroxidase coupled goat anti-mouse antibody and the ECL™ (Amersham) detection 10 system.

6.1.8. RADIOIODINATION OF VEGF

Recombinant human VEGF (5 µg; generously provided by Dr. H. Weich) was dissolved in 110 µl sodium phosphate 15 buffer pH 7.6, and iodinated by the procedure of Hunter and Greenwood (1962). The reaction products were separated from the labeled protein by passage over a sephadex G50 column, pre-equilibrated with phosphate buffered saline (PBS) containing 0.7% bovine serum 20 albumin (BSA), and aliquots of the collected fractions were counted before and after precipitation with 20% trichloroacetic acid. The purity of the iodinated product was estimated to be superior to 90%, as determined by gel 25 electrophoresis, and the specific activity was 77000 cpm/ng. The bioactivity of the iodinated VEGF was confirmed by comparison with the bioactivities of native VEGF using the tissue factor introduction assay described by Clauss, M. et al. (1990 J. Exp. Med. 172:1535-1545).

30 6.1.9. CROSSLINKING OF VEGF TO Flk-1

COS-1 cells transiently expressing Flk-1 and untransfected COS-1 cells were incubated with 200 pM ¹²⁵I-VEGF at 4°C overnight, then washed twice with PBS and exposed to 0.5 mM disuccinimidyl suberate (DSS) in PBS 35 for 1 h at 4°C. The cells were lysed, Flk-1

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immunoprecipitated, and analyzed by electrophoresis on a 7% polyacrylamide gel followed by autoradiography.

6.1.10. VEGF BINDING

5 Ligand binding experiments were performed as described previously (Schumacher, R. et al., 1991, J. Biol. Chem. 266:19288-19295), COS-1 cells were grown in a 15-cm culture dish in DMEM for 48h after transfection. Cells were then washed carefully with PBS and incubated
10 with 5 ml of 25 mM EDTA in PBS for 10 min. Cells were then removed from the plate, washed once with binding buffer (DMEM, 25 mM HEPES, pH 7.5, 0.15% gelatin) and resuspended in 5 ml of binding buffer to determine the cell number. In a total volume of 500 μ l this cell
15 suspension was incubated for 90 min at 15°C with 10 pM 125 I-VEGF, and increasing concentration of unlabeled ligand (from 0 to 7×10^{-9}), which was partially purified from conditioned media of COS-1 cells transiently expressing VEGF (164 amino acid form; Breier et al., 1992). After
20 incubation, cells were washed with PBS 0.1% PBS in the cold. Free ligand was removed by repeated centrifugation and resuspension in binding buffer. Finally, the 125 I radioactivity bound to the cells were determined in a gamma counter (Riastar). Data obtained were analyzed by
25 the method of Munson, P.J. and Rodbard, D. (1980 Anal. Biochem. 107:220-235).

6.1.11. RETROVIRAL VECTORS ENCODING TRANSDOMINANT-NEGATIVE MUTANTS OF Flk-1

30 Recombinant retroviral vectors were constructed that contained the coding region for amino acids 1 through 806 of the Flk-1 receptor (pLX Flk-1 cl.1 and cl.3, Figure 12). A recombinant virus containing a truncated c-fms
35 receptor mutant (pNTK cfms TM cl.7) was used as a control. To obtain virus producing cells mouse GPE cells

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were infected with amphotrophic virus-containing conditioned media of PA317 cells that had been transfected with recombinant retroviral DNA. C6 glioblastoma tumor cells were implanted into nude mice 5 either alone or coimplanted with virus producing cells. Injected cell numbers for the two sets of experiments are indicated below. Beginning at the time when the first tumors appeared, tumor volumes were measured every 2 to 3 days to obtain a growth curve.

10

Experiment No. 1

| | Number of Mice | Number of C6 Cells | Virus-Producer Cell Line | Number of Virus-Cells |
|----|----------------|--------------------|--------------------------|-----------------------|
| 15 | 4 | 5×10^3 | PLXSN Flk-1 TM cl.3 | 1×10^7 |
| | 4 | 5×10^3 | None | 0 |
| | 4 | 5×10^3 | PNTK cfms TM cl.7 | 5×10^6 |

Experiment No. 2

| | Number of Mice | Number of C6 Cells | Virus-Producer Cell Line | Number of Virus-Cells |
|----|----------------|--------------------|--------------------------|-----------------------|
| 20 | 4 | 2×10^6 | PLXSN Flk-1 TM cl.1 | 2×10^7 |
| | 4 | 2×10^6 | PLXSN Flk-1 TM cl.3 | 2×10^7 |
| | 4 | 2×10^6 | None | 0 |
| 25 | 4 | 2×10^6 | PNTK cfms TM cl.7 | 2×10^7 |

6.2. RESULTS6.2.1. ISOLATION OF Flk-1

To identify RTKs that are expressed during mouse 30 development, PCR assays using two degenerate oligonucleotide primer pools that were designed on the basis of highly conserved sequences within the kinase domain of RTKs were performed (Hanks, S.K. et al. 1988, Science 241:42-52). DNA extracted from a λgt10 cDNA 35 library of day 8.5 mouse embryos (Fahrner, K. et al.,

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1987, EMBO J., 6:1497-1508), a stage in mouse development at which many differentiation processes begin was used as the template in the PCR assays. In a parallel approach, with the intention of identifying RTKs 5 that regulate angiogenesis, similar primers were used for the amplification of RTK cDNA sequences from capillary endothelial cells that had been isolated from the brains of postnatal day 4-8 mice, a time at which brain endothelial cell proliferation is maximal (Robertson, 10 P.L. et al., 1985, Devel. Brain Res. 23:219-223). Both approaches yielded cDNA sequences (FIG. 11, SEQ. ID NO.:) encoding the recently described fetal liver RTK, Flk-1 (Matthews, W. et al., 1991, Proc. Natl. Acad. Sci. U.S.A. 88:9026-9030). Based on amino acid homology, this 15 receptor is a member of the type III subclass of RTKs (Ullrich, A. and Schlessinger, J. 1990, Cell 61:203-212) and is closely related to human flt, which also contains seven immunoglobulin-like repeats in its extracellular domain in contrast to other RTKs of that subfamily, which 20 contain only five such repeat structures (Matthews, W. et al., 1991, Proc. Natl. Acad. Sci. U.S.A. 88:9026-9030). Sequence comparisons of Flk-1 with KDR (Terman, B.I. et al., 1991, Oncogene 6:1677-1683) and TKr-C (Sarzani, R. et al., 1992, Biochem. Biophys. Res. Comm. 186:706-714) 25 suggest that these are the human and rat homologues of Flk-1, respectively (Figure 1).

6.2.2 EXPRESSION OF Flk-1 mRNA DURING EMBRYONIC DEVELOPMENT

30 As a first step towards the elucidation of the biological function of Flk-1, the expression of Flk-1 mRNA was analyzed in mouse embryos at different development stages. Northern blot hybridization experiments indicated abundant expression of a major 5.5 35 kb mRNA between day 9.5 and day 18.5, with an apparent

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decline towards the end of gestation (Figure 2A). In postnatal day 4-8 brain capillaries Flk-1 mRNA was found to be highly enriched compared to total brain mRNA (Figure 2B).

5 *In situ* hybridization experiments were performed to obtain more detailed information about the expression of Flk-1 during different embryonal stages. A single-stranded antisense, 2619-nucleotide-long DNA probe comprising the Flk-1 extracellular domain was used as a
10 probe because it generated the most specific hybridization signals. As an example, a parasagittal section of a day 14.5 embryo is shown in Figure 3. High levels of hybridization were detected in the ventricle of the heart, the lung, and the meninges; other tissues such
15 as brain, liver, and mandible appeared to contain fewer cells expressing Flk-1 mRNA. Thin strands of Flk-1 expression were also observed in the intersegmental regions of the vertebrae and at the inner surface of the atrium and the aorta. Higher magnification revealed that
20 the expression of Flk-1 seemed to be restricted to capillaries and blood vessels. Closer examination of the heart, for example, showed positive signals only in the ventricular capillaries and endothelial lining of the atrium (Figure 4A). In the lung, Flk-1 expression was
25 detected in peribronchial capillaries, but was absent from bronchial epithelium (Figure 4D). The aorta showed strong hybridization in endothelial cells, but not in the muscular layer (Figure 4C).

30 **6.2.3. EXPRESSION OF FLK-1 DURING ORGAN ANGIOGENESIS**

The neuroectoderm in the telencephalon of a day 11.5 mouse embryo is largely avascular; the first vascular sprouts begin to radially invade the organ originating from the perineural vascular plexus (Bär, J., 1980, *Adv. 35 Anat. Embryol. Cell. Biol.* 59:1-62; Risau, W. and Lemmon,

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V. 1988, Dev. Biol. 125:441-450). At this stage, expression of Flk-1 was high in the perineural vascular plexus and in invading vascular sprouts, as shown in Figure 5A. These *in situ* hybridization analyses indicated that the 5 proliferating endothelial cells of an angiogenic sprout expressed the Flk-1 mRNA. At day 14.5, when the neuroectoderm is already highly vascularized, numerous radial vessels as well as branching vessels of the intraneural plexus contained large amounts of Flk-1 mRNA 10 (Figure 5B). At postnatal day 4, when sprouting and endothelial cell proliferation is at its highest, strong expression of Flk-1 mRNA was observed in endothelial cells (Figure 5C). Conversely, in the adult brain when angiogenesis has ceased, Flk-1 expression was very low 15 (Figure 5D) and appeared to be restricted mainly to the choroid plexus (Figure 6). In the choroid plexus, cells in the inner vascular layer expressed Flk-1 mRNA, while epithelial cells did not (Figure 6A, B).

The embryonic kidney is vascularized by an 20 angiogenic process (Ekblom, P. et al., 1982, Cell Diff. 11:35-39). Glomerular and peritubular capillaries develop synchronously with epithelial morphogenesis. In the postnatal day 4 kidney, in addition to other capillaries, prominent expression of Flk-1 was observed 25 in the presumptive glomerular capillaries (Figure 7A). This expression persisted in the adult kidney (Figure 7C and D) and then seemed to be more confined to the glomerular compared to the early postnatal kidney.

30 6.2.4. Flk-1 EXPRESSION IN
ENDOTHELIAL CELL PROGENITORS

To investigate the possible involvement of Flk-1 in the early stages of vascular development, analysis of embryos at different stages during blood island formation 35 were performed. In a sagittal section of the deciduum of

a day 8.5 mouse embryo, Flk-1 expression was detected on maternal blood vessels in the deciduum, in the yolk sac and in the trophectoderm. Flk-1 mRNA was also found in the allantois and inside the embryo, mainly located in 5 that part where mesenchyma is found (Figure 8A). At a higher magnification of the maternal deciduum, high levels of Flk-1 mRNA expression were found in the inner lining of blood vessels, which consist of endothelial cells (Figure 8B). In the yolk sac, hybridization 10 signals were confined to the mesodermal layer, in which the hemangioblasts differentiate (Figure 8C). Figure 8D shows a blood island at higher magnification, in which the peripheral angioblasts expressed a high level of Flk-1 mRNA.

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6.2.5. Flk-1 IS A HIGH AFFINITY RECEPTOR FOR VEGF

Detailed examination of *in situ* hybridization results and comparison with those for VEGF recently reported by Breier, G. et al. (1992, *Development* 114:521-20 532) revealed a remarkable similarity in expression pattern. Furthermore, Flk-1 expression in the glomerular endothelium and VEGF in the surrounding epithelial cells (Breier, G. et al., 1992, *Development* 114:521-532) raised the possibility of a paracrine relationship between these 25 cells types and suggested therefore a ligand-receptor relationship for VEGF and Flk-1, respectively. In order to test this hypothesis, the full-length Flk-1 cDNA was cloned into the mammalian expression vector pCMV, which contains transcriptional control elements of the human 30 cytomegalovirus (Gorman, C.M. et al., 1989, *Virology* 171:377-385). For transient expression of the receptor, the Flk-1 expressing plasmid was then transfected into COS-1 fibroblasts.

Specific binding of VEGF to the Flk-1 RTK was 35 demonstrated by crosslinking and competition binding

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experiments. Purified 125 I-labeled VEGF was incubated with COS-1 cells transfected with the pCMV-Flk-1 expression vector. Crosslinking with DSS and subsequent analysis of immunoprecipitation, PAGE, and autoradiography revealed 5 an approximately 220 kD band which was not detected in the control experiment with untransfected COS-1 cells and is likely to represent the VEGF/Flk-1 receptor complex (Figure 9A). In addition, VEGF competed with 125 I-VEGF binding to Flk-1 expressing COS-1 cells (Figure 9B), 10 whereas untransfected COS-1 cells did not bind 125 I-VEGF. The interaction of VEGF with the receptor on transfected cells was specific, as PDGF-BB did not compete with binding of 125 I-VEGF. Analysis of the binding data 15 revealed a K_d of about 10^{-10} M, suggesting that Flk-1 is a high affinity receptor of VEGF. This finding, together with the Flk-1 and VEGF *in situ* hybridization results 20 strongly suggests that Flk-1 is a physiologically relevantly receptor for VEGF.

An autophosphorylation assay was performed to 25 confirm the biological relevance of VEGF binding to the Flk-1 receptor. COS1 cells which transiently expressed Flk-1 were starved in DMEM containing 0.5% fetal calf serum for 24h, stimulated with 0.5 mM VEGF, and lysed. The receptors were immunoprecipitated with the Flk-1 30 specific polyclonal antibody CT128, and then analyzed by SDS-PAGE and subsequent immunoblotting using the antiphosphotyrosine antibody 5E2 (Fendly, B.M. et al., 1990, Cancer Research 50:1550-1558). As shown in Figure 10, VEGF stimulation of Flk-1 expressing cells led to a significant induction of tyrosine phosphorylation of the 180 kD Flk-1 receptor.

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6.2.6. INHIBITION OF TUMOR GROWTH BY
TRANSDOMINANT-NEGATIVE INHIBITION OF Flk-1

The Flk-1 receptor is believed to play a major role in vasculogenesis and angiogenesis. Therefore, 5 inhibition of Flk-1 activity may inhibit vasculogenesis of a developing tumor and inhibit its growth. To test this hypothesis, tumor cells (C6 rat glioblastoma) and mouse cells producing a recombinant retrovirus encoding a truncated Flk-1 receptor were mixed and implanted 10 subcutaneously into nude mice. The implanted C6 glioblastoma cells secrete VEGF which will bind to and activate the Flk-1 receptors expressed on the surface of mouse endothelial cells. In the absence of any inhibitors of vasculogenesis, the endothelial cells will 15 proliferate and migrate towards the tumor cells. Alternatively, if at the time of injection, the tumor cells are co-injected with cells producing recombinant retrovirus encoding the dominant-negative Flk-1, the 20 endothelial cells growing towards the implanted tumor cells will become infected with recombinant retrovirus which may result in dominant-negative Flk-1 mutant expression and inhibition of endogenous Flk-1 signaling. Suppression of endothelial cell proliferation and 25 migration will result in failure of the implanted tumor cells to become vascularized which will lead to inhibition of tumor growth. As shown in Figures 12 and 13, tumor growth is significantly inhibited in mice receiving implantations of cells producing truncated Flk-1 indicating that expression of a truncated Flk-1 30 receptor can act in a dominant-negative manner to inhibit the activity of endogenous wild-type Flk-1.

The present invention is not to be limited in scope by the exemplified embodiments which are intended as 35 illustrations of single aspects of the invention, and any clones, DNA or amino acid sequences which are

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functionally equivalent are within the scope of the invention. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the 5 foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

It is also to be understood that all base pair sizes given for nucleotides are approximate and are used for 10 purposes of description.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Ullrich, et al

(ii) TITLE OF INVENTION: FIK-1 IS A RECEPTOR FOR VASCULAR
ENDOTHELIAL GROWTH FACTOR

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:

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(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
 (B) COMPUTER: IBM PC compatible
 (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

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(A) APPLICATION NUMBER: To be assigned
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(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Coruzzi, Laura A.
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5470 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: unknown
 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

(A) NAME/KEY: CDS
 (B) LOCATION: 286..4386

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

| | |
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| TATAGGGCGA ATTGGGTACG GGACCCCCCT CGAGGTCGAC GGTATCGATA AGCTTGATAT | 60 |
| CGAATTCCGGG CCCAGACTGT GTCCCCCAGC CGGGATAACC TGGCTGACCC GATTCCGGGG | 120 |
| ACACCGCTGA CAGCCGCCGC TGGAGCCAGG GCGCCGGTGC CCCGCGCTCT CCCCCGTCTT | 180 |
| GGGCTGGGGG CGCCATACCG CCTCTGTGAC TTCTTTCGGG CCCAGGGACG GAGAAGGAGT | 240 |

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| | |
|-------------------------------------------------------------------------------------------------------------------------------------------------------|------|
| CTGTGCCTGA GAAACTGGGC TCTGTCCCCA GCGGGAGGT CCAGG ATG GAC AGC Met Glu Ser 1 | 294 |
| AAG GCG CTG CTA GCT GTC CTG TGG TTC TGC GTG GAG ACC CGA GCC Lys Ala Leu Leu Ala Val Ala Leu Trp Phe Cys Val Glu Thr Arg Ala 5 10 15 | 342 |
| GCC TCT GTG GGT TTG ACT GGC GAT TTT CTC CAT CCC CCC AAG CTC AGC Ala Ser Val Gly Leu Thr Gly Asp Phe Leu His Pro Pro Lys Leu Ser 20 25 30 35 | 390 |
| ACA CAG AAA GAC ATA CTG ACA ATT TTG GCA AAT ACA ACC CTT CAG ATT Thr Gln Lys Asp Ile Leu Thr Ile Leu Ala Asn Thr Thr Leu Gln Ile 40 45 50 | 438 |
| ACT TGC AGG GGA CAG CGG GAC CTG GAC TGG CTT TGG CCC AAT GCT CAG Thr Cys Arg Gly Gln Arg Asp Leu Asp Trp Leu Trp Pro Asn Ala Gln 55 60 65 | 486 |
| CGT GAT TCT GAG GAA AGG GTA TTG GTG ACT GAA TGC CCC GGT GGT GAC Arg Asp Ser Glu Glu Arg Val Leu Val Thr Glu Cys Gly Gly Gly Asp 70 75 80 | 534 |
| AGT ATC TTC TGC AAA ACA CTC ACC ATT CCC AGG GTG GTT GGA AAT GAT Ser Ile Phe Cys Lys Thr Leu Thr Ile Pro Arg Val Val Gly Asn Asp 85 90 95 | 582 |
| ACT GGA GCC TAC AAG TGC TCG TAC CGG GAC GTC GAC ATA GCC TCC ACT Thr Gly Ala Tyr Lys Cys Ser Tyr Arg Asp Val Asp Ile Ala Ser Thr 100 105 110 115 | 630 |
| GTT TAT GTC TAT GTT CGA GAT TAC AGA TCA CCA TTC ATC GCC TCT GTC Val Tyr Val Tyr Val Arg Asp Tyr Arg Ser Pro Phe Ile Ala Ser Val 120 125 130 | 678 |
| AGT GAC CAG CAT GCC ATC GTG TAC ATC ACC GAG AAC AAC AAC AAA ACT Ser Asp Gln His Gly Ile Val Tyr Ile Thr Glu Asn Lys Asn Lys Thr 135 140 145 | 726 |
| GTG GTG ATC CCC TGC CGA GGG TCG ATT TCA AAC CTC AAT GTG TCT CTT Val Val Ile Pro Cys Arg Gly Ser Ile Ser Asn Leu Asn Val Ser Leu 150 155 160 | 774 |
| TGC CCT AGG TAT CCA CAA AAG AGA TTT GTT CCG GAT GGA AAC AGA ATT Cys Ala Arg Tyr Pro Glu Lys Arg Phe Val Pro Asp Gly Asn Arg Ile 165 170 175 | 822 |
| TCC TGG GAC AGC GAC ATA GGC TTT ACT CTC CCC AGT TAC ATG ATC AGC Ser Trp Asp Ser Glu Ile Gly Phe Thr Leu Pro Ser Tyr Met Ile Ser 180 185 190 195 | 870 |
| TAT GCC GGC ATG GTC TTC TGT GAG GCA AAG ATC AAT GAT GAA ACC TAT Tyr Ala Gly Met Val Phe Cys Glu Ala Lys Ile Asn Asp Glu Thr Tyr 200 205 210 | 918 |
| CAG TCT ATC ATG TAC ATA GTT GTG GTT GTC GAA TAT AGG ATT TAT GAT Gln Ser Ile Met Tyr Ile Val Val Val Gly Tyr Arg Ile Tyr Asp 215 220 225 | 966 |
| GTG ATT CTG AGC CCC CCG CAT GAA ATT GAG CTA TCT GCC GGA GAA AAA Val Ile Leu Ser Pro Pro His Glu Ile Glu Leu Ser Ala Gly Glu Lys 230 235 240 | 1014 |
| CTT GTC TTA AAT TGT ACA GCG AGA ACA GAG CTC AAT GTG GGG CTT GAT Leu Val Leu Asn Cys Thr Ala Arg Thr Glu Leu Asn Val Gly Leu Asp 245 250 255 | 1062 |

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|-------------------------------------------------------------------------------------------------------------------------------------------------------|------|
| TTC ACC TGG CAC TCT CCA CCT TCA AAG TCT CAT CAT AAG AAG ATT GTC Phe Thr Trp His Ser Pro Pro Ser Lys Ser His His Lys Lys Ile Val 260 265 270 275 | 1110 |
| AAC CGG GAT GTG AAA CCC TTT CCT GGG ACT GTG CGG AAG ATG TTT TTG Asn Arg Asp Val Lys Pro Phe Pro Gly Thr Val Ala Lys Met Phe Leu 280 285 290 | 1158 |
| AGC ACC TTG ACA ATA GAA AGT GTG ACC AAG AGT GAC CAA GGG CAA TAC Ser Thr Leu Thr Ile Glu Ser Val Thr Lys Ser Asp Gln Gly Glu Tyr 295 300 305 | 1206 |
| ACC TGT GTA CGG TCC AGT CGA CGG ATG ATC AAG AGA AAT AGA ACA TTT Thr Cys Val Ala Ser Ser Gly Arg Met Ile Lys Arg Asn Arg Thr Phe 310 315 320 | 1254 |
| GTC CGA GTT CAC ACA AAG CCT TTT ATT GCT TTC GGT AGT GGG ATG AAA Val Arg Val His Thr Lys Pro Phe Ile Ala Phe Gly Ser Gly Met Lys 325 330 335 | 1302 |
| TCT TTG GTG GAA GCC ACA GTG GGC AGT CAA GTC CGA ATC CCT GTG AAG Ser Leu Val Glu Ala Thr Val Gly Ser Gln Val Arg Ile Pro Val Lys 340 345 350 355 | 1350 |
| TAT CTC AGT TAC CCA CCT GAT ATC AAA TGG TAC AGA AAT GGA AGG Tyr, Leu, Ser Tyr, Pro Ala Pro Asp, Ile Lys, Trp, Tyr Arg Asn Gly Arg 360 365 370 | 1398 |
| CCC ATT GAG TCC AAC TAC ACA ATG ATT GTT GGC GAT GAA CTC ACC ATC Pro Ile Glu Ser Asn Tyr Thr Met Ile Val Gly Asp Glu Leu Thr Ile 375 380 385 | 1446 |
| ATG GAA GTG ACT GAA AGA GAT GCA GGA AAC TAC ACG GTC ATC CTC ACC Met Glu Val Thr Glu Arg Asp Ala Gly Asn Tyr Thr Val Ile Leu Thr 390 395 400 | 1494 |
| AAC CCC ATT TCA ATG GAG AAA CAG AGC CAC ATG GTC TCT CTG GTT GTG Asn Pro Ile Ser Met Glu Lys Gln Ser His Met Val Ser Leu Val Val 405 410 415 | 1542 |
| AAT GTC CCA CCC CAG ATC GGT GAG AAA GCC TTG ATC TCG CCT ATG GAT Asn Val Pro Pro Gln Ile Gly Glu Lys Ala Leu Ile Ser Pro Met Asp 420 425 430 435 | 1590 |
| TCC TAC CAG TAT GGG ACC ATG CAG ACA TTG ACA TGC ACA GTC TAC CCC Ser Tyr Gln Tyr Gly Thr Met Gln Thr Leu Thr Cys Thr Val Tyr Ala 440 445 450 | 1638 |
| AAC CCT CCC CTG CAC CAC ATC CAG TGG TAC TGG CAG CTA GAA GAA GCC Asn Pro Pro Leu His His Ile Gln Trp Tyr Trp Gln Leu Glu Glu Ala 455 460 465 | 1686 |
| TGG TCC TAC AGA CCC GGC CAA ACA AGC CCG TAT GCT TGT AAA GAA TGG Cys Ser Tyr Arg Pro Gly Gln Thr Ser Pro Tyr Ala Cys Lys Glu Trp 470 475 480 | 1734 |
| AGA CAC GTG GAG GAT TTC CAG GGG GGA AAC AAG ATC GAA GTC ACC AAA Arg His Val Glu Asp Phe Gln Gly Gly Asn Lys Ile Glu Val Thr Lys 485 490 495 | 1782 |
| AAC CAA TAT GCC CTG ATT GAA GCA AAA AAC AAA ACT GTC AGT ACG CTG Asn Gln Tyr Ala Leu Ile Glu Gly Lys Asn Lys Thr Val Ser Thr Leu 500 505 510 515 | 1830 |
| GTC ATC CAA GCT GCC AAC GTG TCA GCG TTG TAC AAA TGT GAA GCC ATC Val Ile Gln Ala Ala Asn Val Ser Ala Leu Tyr Lys Cys Glu Ala Ile 520 525 530 | 1878 |

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|-------------------------------------------------------------------------------------------------------------------------------------------------------|------|
| AAC AAA GCG CGA CGA CGA GAG AGG GTC ATC TCC TTC CAT GTG ATC AGG Asn Lys Ala Gly Arg Gly Glu Arg Val Ile Ser Phe His Val Ile Arg 535 540 545 | 1926 |
| GCT CCT GAA ATT ACT GTG CAA CCT CCT CCC CAG CCA ACT GAG CAG GAG Gly Pro Glu Ile Thr Val Gln Pro Ala Ala Gln Pro Thr Glu Gln Glu 550 555 560 | 1974 |
| AGT GTG TCC CTG TTG TGC ACT GCA GAC AGA AAT ACG TTT GAG AAC CTC Ser Val Ser Leu Leu Cys Thr Ala Asp Arg Asn Thr Phe Glu Asn Leu 565 570 575 | 2022 |
| ACG TGG TAC ARG CTT GGC TCA CAG GCA ACA TCG GTC CAC ATG GGC GAA Thr Trp Tyr Lys Leu Gly Ser Gln Ala Thr Ser Val His Met Gly Glu 580 585 590 595 | 2070 |
| TCA CTC ACA CCA GTT TGC AAG AAC TTG GAT GCT CTT TGG AAA CTG AAT Ser Leu Thr Pro Val Cys Lys Asn Leu Asp Ala Leu Trp Lys Leu Asn 600 605 610 | 2118 |
| GCG ACC ATG TTT TCT AAC AGC ACA AAT GAC ATC TTG ATT GTG GCA TTT Gly Thr Met Phe Ser Asn Ser Thr Asn Asp Ile Leu Ile Val Ala Phe 615 620 625 | 2166 |
| CAG AAT GCC TCT CTG CAG GAC CAA GGC GAC TAT GTT TGC TCT GCT CAA Gln Asn Ala Ser Leu Gln Asp Gln Gly Asp Tyr Val Cys Ser Ala Gln 630 635 640 | 2214 |
| GAT AAG AAG ACC AAG AAA AGA CAT TGC CTG GTC AAA CAG CTC ATC ATC Asp Lys Lys Thr Lys Lys Arg His Cys Leu Val Lys Gln Leu Ile Ile 645 650 655 | 2262 |
| CTA GAG CGC ATG GCA CCC ATG ATC ACC GCA AAT CTG GAG AAT CAG ACA Leu Glu Arg Met Ala Pro Met Ile Thr Gly Asn Leu Glu Asn Gln Thr 660 665 670 675 | 2310 |
| ACA ACC ATT GGC GAG ACC ATT GAA GTG ACT TGC CCA GCA TCT GGA AAT Thr Thr Ile Gly Glu Thr Ile Glu Val Thr Cys Pro Ala Ser Gly Asn 680 685 690 | 2358 |
| CCT ACC CCA CAC ATT ACA TGG TTC AAA GAC AAC GAG ACC CTG GTC GAA Pro Thr Pro His Ile Thr Trp Phe Lys Asp Asn Glu Thr Leu Val Glu 695 700 705 | 2406 |
| GAT TCA GGC ATT GTC CTG AGA GAT GGG AAC CCG AAC CTG ACT ATC CGC Asp Ser Gly Ile Val Leu Arg Asp Gly Asn Arg Asn Leu Thr Ile Arg 710 715 720 | 2454 |
| AGG GTG AGG AAG GAG GAT GGA GGC CTC TAC ACC TGC CGG GCC TGC AAT Arg Val Arg Lys Glu Asp Gly Gly Leu Tyr Thr Cys Gln Ala Cys Asn 725 730 735 | 2502 |
| GTC CTT GGC TGT GCA AGA GCG GAG ACG CTC TTC ATA ATA GAA GGT GCC Val Leu Gly Cys Ala Arg Ala Glu Thr Leu Phe Ile Ile Glu Gly Ala 740 745 750 755 | 2550 |
| CAG GAA AAG ACC AAC TTG GAA GTC ATT ATC CTC GTC GGC ACT GCA GTG Gln Glu Lys Thr Asn Leu Glu Val Ile Ile Leu Val Gly Thr Ala Val 760 765 770 | 2598 |
| ATT GCC ATG TTC TGG CTC CTT CTT GTC ATT GTC CTA CGG ACC GTT Ile Ala Met Phe Phe Trp Leu Leu Leu Val Ile Val Leu Arg Thr Val 775 780 785 | 2646 |
| AAG CGG CCC AAT GAA GGG GAA CTG AAG ACA GCA TAC TTG TCT ATT GTC Lys Arg Ala Asn Glu Gly Glu Leu Lys Thr Gly Tyr Leu Ser Ile Val 790 795 800 | 2694 |

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|-----------------------------------------------------------------------------------------------------------------------------------------------------------|------|
| ATG GAT CCA GAT GAA TTG CCC TTG GAT GAG CGC TGT GAA CCC TTG CCT Met Asp Pro Asp Glu Leu Pro Leu Asp Glu Arg Cys Glu Arg Leu Pro 805 810 815 | 2742 |
| TAT GAT GCC AGC AAG TGG GAA TTC CCC AGG GAC CGG CTG AAA CTA GGA Tyr Asp Ala Ser Lys Trp Glu Phe Pro Arg Asp Arg Leu Lys Leu Gly 820 825 830 835 | 2790 |
| AAA CCT CTT CGC CGC GGT GCC TTC CGC CAA GTG ATT GAG GCA GAC GCT Lys Pro Leu Gly Arg Gly Ala Phe Gly Gln Val Ile Glu Ala Asp Ala 840 845 850 | 2838 |
| TTT GGA ATT GAC AAG ACA GCG ACT TGC AAA ACA GTC GCC GTC AAG ATG Phe Gly Ile Asp Lys Thr Ala Thr Cys Lys Thr Val Ala Val Lys Met 855 860 865 | 2886 |
| TTG AAA GAA GGA GCA ACA CAC AGC GAG CAT CGA GCC CTC ATG TCT GAA Leu Lys Glu Gly Ala Thr His Ser Glu His Arg Ala Leu Met Ser Glu 870 875 880 | 2934 |
| CTC AAG ATC CTC ATC CAC ATT GGT CAC CAT CTC AAT GTG GTG AAC CTC Leu Lys Ile Leu Ile His Ile Gly His His Leu Asn Val Val Asn Leu 885 890 895 | 2982 |
| CTA CGC GCC TGC ACC AAG CCG GGA GGG CCT CTC ATG GTG ATT GTG GAA Leu Gly Ala Cys Thr Lys Pro Gly Pro Leu Met Val Ile Val Glu 900 905 910 915 | 3030 |
| TTC TGC AAG TTT GGA AAC CTA TCA ACT TAC TTA CGG GGC AAG AGA AAT Phe Cys Lys Phe Gly Asn Leu Ser Thr Tyr Leu Arg Gly Lys Arg Asn 920 925 930 | 3078 |
| GAA TTT GTT CCC TAT AAG AGC AAA GGG GCA CGC TTC CGC CAG GGC AAG Glu Phe Val Pro Tyr Lys Ser Lys Gly Ala Arg Phe Arg Gln Gly Lys 935 940 945 | 3126 |
| GAC TAC GTT GGG GAG CTC TCC GTG GAT CTC AAA AGA CGC TTG GAC AGC Asp Tyr Val Gly Glu Leu Ser Val Asp Leu Lys Arg Arg Leu Asp Ser 950 955 960 | 3174 |
| ATC ACC AGC AGC CAG AGC TCT GCC AGC TCA CGC TTT GTT GAG GAG AAA Ile Thr Ser Ser Gln Ser Ser Ala Ser Ser Gly Phe Val Glu Glu Lys 965 970 975 | 3222 |
| TCG CTC ACT GAT GTC GAG GAA GAA GAA GCT TCT GAA GAA CTG TAC AAG Ser Leu Ser Asp Val Glu Glu Glu Ala Ser Glu Glu Leu Tyr Lys 980 985 990 995 | 3270 |
| GAC TTC CTG ACC TTG GAG CAT CTC ATC TGT TAC AGC TTC CAA GTG GCT Asp Phe Leu Thr Leu Glu His Leu Ile Cys Tyr Ser Phe Gln Val Ala 1000 1005 1010 | 3318 |
| AAG GGC ATG GAG TTC TTG GCA TCA AGG AAG TGT ATC CAC AGG GAC CTG Lys Gly Met Glu Phe Leu Ala Ser Arg Lys Cys Ile His Arg Asp Leu 1015 1020 1025 | 3366 |
| GCA GCA CGA AAC ATT CTC CTA TCG GAG AAG AAT GTG GTT AAG ATC TGT Ala Ala Arg Asn Ile Leu Leu Ser Glu Lys Asn Val Val Lys Ile Cys 1030 1035 1040 | 3414 |
| GAC TTC GGC TTG GCC CGG GAC ATT TAT AAA GAC CCG GAT TAT GTC AGA Asp Phe Gly Leu Ala Arg Asp Ile Tyr Lys Asp Pro Asp Tyr Val Arg 1045 1050 1055 | 3462 |
| AAA GGA GAT GCC CGA CTC CCT TTG AAG TGG ATG GCC CCG GAA ACC ATT Lys Gly Asp Ala Arg Leu Pro Leu Lys Trp Met Ala Pro Glu Thr Ile 1060 1065 1070 1075 | 3510 |

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|-----------------------------------------------------------------------------------------------------------------------------------------------------------|------|
| TTT GAC AGA GTA TAC ACA ATT CAG AGC GAT GTG TCG TCT TTC CGT GTG Phe Asp Arg Val Tyr Thr Ile Gln Ser Asp Val Trp Ser Phe Gly Val 1080 1085 1090 | 3558 |
| TTG CTC TGG GAA ATA TTT TCC TTA GGT GCC TCC CCA TAC CCT GGG GTC Leu Leu Trp Glu Ile Phe Ser Leu Gly Ala Ser Pro Tyr Pro Gly Val 1095 1100 1105 | 3606 |
| AAG ATT GAT GAA GAA TTT TGT AGG AGA TTG AAA GAA GGA ACT AGA ATG Lys Ile Asp Glu Glu Phe Cys Arg Arg Leu Lys Glu Gly Thr Arg Met 1110 1115 1120 | 3654 |
| CGG GCT CCT GAC TAC ACT ACC CCA GAA ATG TAC CAG ACC ATG CTG GAC Arg Ala Pro Asp Tyr Thr Pro Glu Met Tyr Gln Thr Met Leu Asp 1125 1130 1135 | 3702 |
| TGC TGG CAT GAG GAC CCC AAC CAG AGA CCC TCG TTT TCA GAG TTG GTG Cys Trp His Glu Asp Pro Asn Gln Arg Pro Ser Phe Ser Glu Leu Val 1140 1145 1150 1155 | 3750 |
| GAG CAT TTG GGA AAC CTC CTG CAA GCA AAT GCG CAG CAG GAT GGC AAA Glu His Leu Gly Asn Leu Leu Gln Ala Asn Ala Gln Gln Asp Gly Lys 1160 1165 1170 | 3798 |
| GAC TAT ATT GTT CTT CCA ATG TCA GAG ACA CTG AGC ATG GAA GAG GAT Asp Tyr Ile Val Leu Pro Met Ser Glu Thr Leu Ser Met Glu Glu Asp 1175 1180 1185 | 3846 |
| TCT GGA CTC TCC CTG CCT ACC TCA CCT GTT TCC TGT ATG GAG GAA GAG Ser Gly Leu Ser Leu Pro Thr Ser Pro Val Ser Cys Met Glu Glu Glu 1190 1195 1200 | 3894 |
| GAA GTG TCC GAC CCC AAA TTC CAT TAT GAC AAC ACA GCA GGA ATC AGT Glu Val Cys Asp Pro Lys Phe His Tyr Asp Asn Thr Ala Gly Ile Ser 1205 1210 1215 | 3942 |
| CAT TAT CTC CAG AAC AGT AAG CGA AAG AGC CGG CCA GTG AGT GTA AAA His Tyr Leu Gln Asn Ser Lys Arg Lys Ser Arg Pro Val Ser Val Lys 1220 1225 1230 1235 | 3990 |
| ACA TTT GAA GAT ATC CCA TTG GAG GAA CCA GAA GTA AAA GTG ATC CCA Thr Phe Glu Asp Ile Pro Leu Glu Glu Pro Glu Val Lys Val Ile Pro 1240 1245 1250 | 4038 |
| GAT GAC AGC CAG ACA GAC AGT CGG ATG GTC CTT GCA TCA GAA GAG CTG Asp Asp Ser Gln Thr Asp Ser Gly Met Val Leu Ala Ser Glu Glu Leu 1255 1260 1265 | 4086 |
| AAA ACT CTG GAA GAC AGG AAC AAA TTA TCT CCA TCT TTT GGT GGA ATG Lys Thr Leu Glu Asp Arg Asn Lys Leu Ser Pro Ser Phe Gly Gly Met 1270 1275 1280 | 4134 |
| ATG CCC AGT AAA AGC AGG GAG TCT GTG GCC TCG GAA GGC TCC AAC CAG Met Pro Ser Lys Ser Arg Glu Ser Val Ala Ser Glu Gly Ser Asn Gln 1285 1290 1295 | 4182 |
| ACC AGT GGC TAC CAG TCT CGG TAT CAC TCA GAT GAC ACA GAC ACC ACC Thr Ser Gly Tyr Gln Ser Gly Tyr His Ser Asp Asp Thr Asp Thr Thr 1300 1305 1310 1315 | 4230 |
| CTG TAC TCC AGC GAC GAG GCA GGA CTT TTA AAG ATG GTG GAT GCT GCA Val Tyr Ser Ser Asp Glu Ala Gly Leu Leu Lys Met Val Asp Ala Ala 1320 1325 1330 | 4278 |
| GTT CAC GCT GAC TCA GGG ACC ACA CTG CAG CTC ACC TCC TGT TTA AAT Val His Ala Asp Ser Gly Thr Thr Leu Gln Leu Thr Ser Cys Leu Asn 1335 1340 1345 | 4326 |

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| | |
|--------------------------------------------------------------------|------|
| GCA AGT GGT CCT GTC CCG GCT CCC CCA ACT CCT GGA AAT CAC GAG | 4374 |
| Gly Ser Gly Pro Val Pro Ala Pro Pro Pro Thr Pro Gly Asn His Glu | |
| 1350 1355 1360 | |
| AGA GGT GCT GCT TAGATTTCA AGTGTGTTG TTTCCACCCAC CCGGAAGTAG | 4426 |
| Arg Gly Ala Ala | |
| 1365 | |
| CCACATTGATTTTGGAGGACGGACCTCAGACTGCAAGGAGCTTGTCCTCAGG | 4486 |
| GCATTTCCAGAGAACATGCCCATGACCCAA GAAATGTTGACTCTACTCTCTTTCATT | 4546 |
| CATTTAAAGTCCTATATAA TGTGCCCTGC TGTGGTCTCA CTACCAAGTTAAAGCAAAAGA | 4606 |
| CTTCAAAACA CGTGGACTCTGTCCTCAGAAGTGGCAACGGCACCTCTG TGAAACTGGA | 4666 |
| TCGAATGGGC AATGCTTGTGTGTGAGA TCGGTGAGAT GTCCCAGGGC CGAGTCTGTC | 4726 |
| TACCTTGGAG GCTTGTGGA GGATGCCGGC TATGAGCCAA GTGTTAAGTG TGGGATGTGG | 4786 |
| ACTGGGAGGA AGGAAGGCCAACGCTCTGGAGAGGCCGTTGGAGCTGCA GATGCATTGT | 4846 |
| GCTGGCTCTG GTGGAGGTGG GCTTGTGCCCTGTCAGGAAACGGCGGCCGGCAGGG | 4906 |
| TTTGGTTTTC GAAAGTTTGC GTGCTCTTCA CACTGGGTTACGGGAGT TCCCTGTGGC | 4966 |
| GTTCCCTACT CCTAATGAGA GTTCCCTCCG GACTCTTACG TGTCTCCTGG CCTGGCCCCA | 5026 |
| CGAAGGAAAT GATGCAGCTT GTCCTTCCCTCATCTCTCAG GCTGTGCCCTT AATTCAAGAAC | 5086 |
| ACCAAAAGAG AGGAACGTGGAGAGGCCCTGACGGGGCGCGAAGAATTG TGAGAACAGA | 5146 |
| ACAGAAACTC AGGGTTCTG CTGGGTGGAG ACCCACGTGGCGCCCTGGTG GCAGGTCTGA | 5206 |
| GGGTTCTCTG TCAAGTGGCG GTAAAGGCTC AGGCTGGTGTCTTCTCTA TCTCCACTCC | 5266 |
| TGTCAAGGCCCTCAGTATTTAG CTTTGTGGCTTCCTGATGGCAGAAAAATCT | 5326 |
| TAATTGGTTG GTTGTCTCAGATAATCA CTAGCCAGAT TTGAAATTACCTTTAGCC | 5386 |
| GAGGTTATGA TAACATCTAC TGTATCCTTT AGAATTTAA CCTATAAAAC TATGTCTACT | 5446 |
| GGTTTCTGCC TGTGTGCTTA TGTT | 5470 |

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1367 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

| | |
|-----------------------------------------------------------------|--|
| Met Glu Ser Lys Ala Leu Leu Ala Val Ala Leu Trp Phe Cys Val Glu | |
| 1 5 10 15 | |
| Thr Arg Ala Ala Ser Val Gly Leu Thr Gly Asp Phe Leu His Pro Pro | |
| 20 25 30 | |
| Lys Leu Ser Thr Gln Lys Asp Ile Leu Thr Ile Leu Ala Asn Thr Thr | |
| 35 40 45 | |
| Leu Gln Ile Thr Cys Arg Gly Gln Arg Asp Leu Asp Trp Leu Trp Pro | |
| 50 55 60 | |

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Asn Ala Gln Arg Asp Ser Glu Glu Arg Val Leu Val Thr Glu Cys Gly
 65 70 75 80
 Gly Gly Asp Ser Ile Phe Cys Lys Thr Leu Thr Ile Pro Arg Val Val
 85 90 95
 Gly Asn Asp Thr Gly Ala Tyr Lys Cys Ser Tyr Arg Asp Val Asp Ile
 100 105 110
 Ala Ser Thr Val Tyr Val Tyr Val Arg Asp Tyr Arg Ser Pro Phe Ile
 115 120 125
 Ala Ser Val Ser Asp Gln His Gly Ile Val Tyr Ile Thr Glu Asn Lys
 130 135 140
 Asn Lys Thr Val Val Ile Pro Cys Arg Gly Ser Ile Ser Asn Leu Asn
 145 150 155 160
 Val Ser Leu Cys Ala Arg Tyr Pro Glu Lys Arg Phe Val Pro Asp Gly
 165 170 175
 Asn Arg Ile Ser Trp Asp Ser Glu Ile Gly Phe Thr Leu Pro Ser Tyr
 180 185 190
 Met Ile Ser Tyr Ala Gly Met Val Phe Cys Glu Ala Lys Ile Asn Asp
 195 200 205
 Glu Thr Tyr Gln Ser Ile Met Tyr Ile Val Val Val Gly Tyr Arg
 210 215 220
 Ile Tyr Asp Val Ile Leu Ser Pro Pro His Glu Ile Glu Leu Ser Ala
 225 230 235 240
 Gly Glu Lys Leu Val Leu Asn Cys Thr Ala Arg Thr Glu Leu Asn Val
 245 250 255
 Gly Leu Asp Phe Thr Trp His Ser Pro Pro Ser Lys Ser His His Lys
 260 265 270
 Lys Ile Val Asn Arg Asp Val Lys Pro Phe Pro Gly Thr Val Ala Lys
 275 280 285
 Met Phe Leu Ser Thr Leu Thr Ile Glu Ser Val Thr Lys Ser Asp Gln
 290 295 300
 Gly Glu Tyr Thr Cys Val Ala Ser Ser Gly Arg Met Ile Lys Arg Asn
 305 310 315 320
 Arg Thr Phe Val Arg Val His Thr Lys Pro Phe Ile Ala Phe Gly Ser
 325 330 335
 Gly Met Lys Ser Leu Val Glu Ala Thr Val Gly Ser Gln Val Arg Ile
 340 345 350
 Pro Val Lys Tyr Leu Ser Tyr Pro Ala Pro Asp Ile Lys Trp Tyr Arg
 355 360 365
 Asn Gly Arg Pro Ile Glu Ser Asn Tyr Thr Met Ile Val Gly Asp Glu
 370 375 380
 Leu Thr Ile Met Glu Val Thr Glu Arg Asp Ala Gly Asn Tyr Thr Val
 385 390 395 400
 Ile Leu Thr Asn Pro Ile Ser Met Glu Lys Gln Ser His Met Val Ser
 405 410 415
 Leu Val Val Asn Val Pro Pro Gln Ile Gly Glu Lys Ala Leu Ile Ser

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420

425

430

Pro Met Asp Ser Tyr Gln Tyr Gly Thr Met Gln Thr Leu Thr Cys Thr
 435 440 445
 Val Tyr Ala Asn Pro Pro Leu His His Ile Gln Trp Tyr Trp Gln Leu
 450 455 460
 Glu Glu Ala Cys Ser Tyr Arg Pro Gly Gln Thr Ser Pro Tyr Ala Cys
 465 470 475 480
 Lys Glu Trp Arg His Val Glu Asp Phe Gln Gly Gly Asn Lys Ile Glu
 485 490 495
 Val Thr Lys Asn Gln Tyr Ala Leu Ile Glu Gly Lys Asn Lys Thr Val
 500 505 510
 Ser Thr Leu Val Ile Gln Ala Ala Asn Val Ser Ala Leu Tyr Lys Cys
 515 520 525
 Glu Ala Ile Asn Lys Ala Gly Arg Gly Glu Arg Val Ile Ser Phe His
 530 535 540
 Val Ile Arg Gly Pro Glu Ile Thr Val Gln Pro Ala Ala Gln Pro Thr
 545 550 555 560
 Glu Gln Glu Ser Val Ser Leu Leu Cys Thr Ala Asp Arg Asn Thr Phe
 565 570 575
 Glu Asn Leu Thr Trp Tyr Lys Leu Gly Ser Gln Ala Thr Ser Val His
 580 585 590
 Met Gly Glu Ser Leu Thr Pro Val Cys Lys Asn Leu Asp Ala Leu Trp
 595 600 605
 Lys Leu Asn Gly Thr Met Phe Ser Asn Ser Thr Asn Asp Ile Leu Ile
 610 615 620
 Val Ala Phe Gln Asn Ala Ser Leu Gln Asp Gln Gly Asp Tyr Val Cys
 625 630 635 640
 Ser Ala Gln Asp Lys Lys Thr Lys Lys Arg His Cys Leu Val Lys Gln
 645 650 655
 Leu Ile Ile Leu Glu Arg Met Ala Pro Met Ile Thr Gly Asn Leu Glu
 660 665 670
 Asn Gln Thr Thr Ile Gly Glu Thr Ile Glu Val Thr Cys Pro Ala
 675 680 685
 Ser Gly Asn Pro Thr Pro His Ile Thr Trp Phe Lys Asp Asn Glu Thr
 690 695 700
 Leu Val Glu Asp Ser Gly Ile Val Leu Arg Asp Gly Asn Arg Asn Leu
 705 710 715 720
 Thr Ile Arg Arg Val Arg Lys Glu Asp Gly Gly Leu Tyr Thr Cys Gln
 725 730 735
 Ala Cys Asn Val Leu Gly Cys Ala Arg Ala Glu Thr Leu Phe Ile Ile
 740 745 750
 Glu Gly Ala Gln Glu Lys Thr Asn Leu Glu Val Ile Ile Leu Val Gly
 755 760 765
 Thr Ala Val Ile Ala Met Phe Phe Trp Leu Leu Val Ile Val Leu
 770 775 780

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Arg Thr Val Lys Arg Ala Asn Glu Gly Glu Leu Lys Thr Gly Tyr Leu
 785 790 795 800
 Ser Ile Val Met Asp Pro Asp Glu Leu Pro Leu Asp Glu Arg Cys Glu
 805 810 815
 Arg Leu Pro Tyr Asp Ala Ser Lys Trp Glu Phe Pro Arg Asp Arg Leu
 820 825 830
 Lys Leu Gly Lys Pro Leu Gly Arg Gly Ala Phe Gly Gln Val Ile Glu
 835 840 845
 Ala Asp Ala Phe Gly Ile Asp Lys Thr Ala Thr Cys Lys Thr Val Ala
 850 855 860
 Val Lys Met Leu Lys Glu Gly Ala Thr His Ser Glu His Arg Ala Leu
 865 870 875 880
 Met Ser Glu Leu Lys Ile Leu Ile His Ile Gly His His Leu Asn Val
 885 890 895
 Val Asn Leu Leu Gly Ala Cys Thr Lys Pro Gly Gly Pro Leu Met Val
 900 905 910
 Ile Val Glu Phe Cys Lys Phe Gly Asn Leu Ser Thr Tyr Leu Arg Gly
 915 920 925
 Lys Arg Asn Glu Phe Val Pro Tyr Lys Ser Lys Gly Ala Arg Phe Arg
 930 935 940
 Gln Gly Lys Asp Tyr Val Gly Glu Leu Ser Val Asp Leu Lys Arg Arg
 945 950 955 960
 Leu Asp Ser Ile Thr Ser Ser Gln Ser Ser Ala Ser Ser Gly Phe Val
 965 970 975
 Glu Glu Lys Ser Leu Ser Asp Val Glu Glu Glu Glu Ala Ser Glu Glu
 980 985 990
 Leu Tyr Lys Asp Phe Leu Thr Leu Glu His Leu Ile Cys Tyr Ser Phe
 995 1000 1005
 Gln Val Ala Lys Gly Met Glu Phe Leu Ala Ser Arg Lys Cys Ile His
 1010 1015 1020
 Arg Asp Leu Ala Ala Arg Asn Ile Leu Leu Ser Glu Lys Asn Val Val
 1025 1030 1035 1040
 Lys Ile Cys Asp Phe Gly Leu Ala Arg Asp Ile Tyr Lys Asp Pro Asp
 1045 1050 1055
 Tyr Val Arg Lys Gly Asp Ala Arg Leu Pro Leu Lys Trp Met Ala Pro
 1060 1065 1070
 Glu Thr Ile Phe Asp Arg Val Tyr Thr Ile Gln Ser Asp Val Trp Ser
 1075 1080 1085
 Phe Gly Val Leu Leu Trp Glu Ile Phe Ser Leu Gly Ala Ser Pro Tyr
 1090 1095 1100
 Pro Gly Val Lys Ile Asp Glu Glu Phe Cys Arg Arg Leu Lys Glu Gly
 1105 1110 1115 1120
 Thr Arg Met Arg Ala Pro Asp Tyr Thr Thr Pro Glu Met Tyr Gln Thr
 1125 1130 1135
 Met Leu Asp Cys Trp His Glu Asp Pro Asn Gln Arg Pro Ser Phe Ser

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| 1140 | 1145 | 1150 |
|-----------------------------------------------------------------|------|------|
| Glu Leu Val Glu His Leu Gly Asn Leu Leu Gln Ala Asn Ala Gln Gln | | |
| 1155 | 1160 | 1165 |
| Asp Gly Lys Asp Tyr Ile Val Leu Pro Met Ser Glu Thr Leu Ser Met | | |
| 1170 | 1175 | 1180 |
| Glu Glu Asp Ser Gly Leu Ser Leu Pro Thr Ser Pro Val Ser Cys Met | | |
| 1185 | 1190 | 1195 |
| Glu Glu Glu Val Cys Asp Pro Lys Phe His Tyr Asp Asn Thr Ala | | |
| 1205 | 1210 | 1215 |
| Gly Ile Ser His Tyr Leu Gln Asn Ser Lys Arg Lys Ser Arg Pro Val | | |
| 1220 | 1225 | 1230 |
| Ser Val Lys Thr Phe Glu Asp Ile Pro Leu Glu Glu Pro Glu Val Lys | | |
| 1235 | 1240 | 1245 |
| Val Ile Pro Asp Asp Ser Gln Thr Asp Ser Gly Met Val Leu Ala Ser | | |
| 1250 | 1255 | 1260 |
| Glu Glu Leu Lys Thr Leu Glu Asp Arg Asn Lys Leu Ser Pro Ser Phe | | |
| 1265 | 1270 | 1275 |
| 1280 | | |
| Gly Gly Met Met Pro Ser Lys Ser Arg Glu Ser Val Ala Ser Glu Gly | | |
| 1285 | 1290 | 1295 |
| Ser Asn Gln Thr Ser Gly Tyr Gln Ser Gly Tyr His Ser Asp Asp Thr | | |
| 1300 | 1305 | 1310 |
| Asp Thr Thr Val Tyr Ser Ser Asp Glu Ala Gly Leu Leu Lys Met Val | | |
| 1315 | 1320 | 1325 |
| Asp Ala Ala Val His Ala Asp Ser Gly Thr Thr Leu Gln Leu Thr Ser | | |
| 1330 | 1335 | 1340 |
| Cys Leu Asn Gly Ser Gly Pro Val Pro Ala Pro Pro Pro Thr Pro Gly | | |
| 1345 | 1350 | 1355 |
| 1360 | | |
| Asn His Glu Arg Gly Ala Ala | | |
| 1365 | | |

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WHAT IS CLAIMED IS:

1. A recombinant DNA vector containing a nucleotide sequence that encodes a Flk-1 operatively associated with a regulatory sequence that controls gene expression in a host.
5
2. A recombinant DNA vector containing a nucleotide sequence that encodes a Flk-1 fusion protein operatively associated with a regulatory sequence that controls gene expression in a host.
10
3. An engineered host cell that contains the recombinant DNA vector of Claims 1 or 2.
15
4. An engineered cell line that contains the recombinant DNA expression vector of Claim 1 and expresses Flk-1.
20
5. The engineered cell line of Claim 3 which expresses the Flk-1 on the surface of the cell.
25
6. An engineered cell line that contains the recombinant DNA expression vector of Claim 2 and expresses the Flk-1 fusion protein.
30
7. The engineered cell line of Claim 6 that expresses the Flk-1 fusion protein on the surface of the cell.
35
8. A method for producing recombinant Flk-1, comprising:
 - (a) culturing a host cell transformed with the recombinant DNA expression vector of Claim 1 and which expresses the Flk-1; and

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(b) recovering the Flk-1 gene product from the cell culture.

9. A method for producing recombinant Flk-1 fusion protein, comprising:

(a) culturing a host cell transformed with the recombinant DNA expression vector of Claim 2 and which expresses the Flk-1 fusion protein; and

10 (b) recovering the Flk-1 fusion protein from the cell culture.

10. An isolated recombinant Flk-1 receptor protein.

15 11. A fusion protein comprising Flk-1 linked to a heterologous protein or peptide sequence.

12. An oligonucleotide which encodes an antisense sequence complementary to a portion of the Flk-1 20 nucleotide sequence, and which inhibits translation of the Flk-1 gene in a cell.

13. The oligonucleotide of Claim 12 which is complementary to a nucleotide sequence encoding the amino 25 terminal region of the Flk-1.

14. A monoclonal antibody which immunospecifically binds to an epitope of the Flk-1.

30 15. The monoclonal antibody of Claim 14 which competitively inhibits the binding of VEGF to the Flk-1.

35 16. The monoclonal antibody of Claim 14 which is linked to a cytotoxic agent.

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17. The monoclonal antibody of Claim 14 which is linked to a radioisotope.

18. A method for screening and identifying 5 antagonists of VEGF, comprising:

(a) contacting a cell line that expresses Flk-1 with a test compound in the presence of VEGF; and

10 (b) determining whether the test compound inhibits the binding and cellular effects of VEGF on the cell line,

in which antagonists are identified as those compounds that inhibit both the binding and cellular effects of VEGF on the cell line.

15

19. A method for screening and identifying agonists of VEGF, comprising:

(a) contacting a cell line that expresses the Flk-1 with a test compound in the presence and in the absence of VEGF;

20 (b) determining whether, in the presence of VEGF, the test compound inhibits the binding of VEGF to the cell line; and

(c) determining whether, in the absence of the VEGF, the test compound mimics the 25 cellular effects of VEGF on the cell line,

in which agonists are identified as those test compounds that inhibit the binding but mimic the cellular effects of VEGF on the cell line.

30

20. The method according to Claims 18 or 19 in which the cell line is a genetically engineered cell line.

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21. The method according to Claims 18 or 19 in which the cell line endogenously expresses the Flk-1.

22. A method for screening and identifying
5 antagonists of VEGF comprising:

- (a) contacting Flk-1 protein with a random peptide library such that Flk-1 will recognize and bind to one or more peptide species within the library;
- 10 (b) isolating the Flk-1/peptide combination;
- (c) determining the sequence of the peptide isolated in step c; and
- (d) determining whether the test compound inhibits the binding and cellular effects
15 of VEGF,

in which antagonists are identified as those peptides that inhibit both the binding and cellular effects of VEGF.

20 23. A method for screening and identifying agonists of VEGF comprising:

- (a) contacting Flk-1 protein with a random peptide library such that Flk-1 will recognize and bind to one or more peptide species within the library;
- 25 (b) isolating the Flk-1/peptide combination;
- (c) determining the sequence of the peptide isolated in step c; and
- (d) determining whether, in the absence of the
30 VRGF, the peptide mimics the cellular effects of VEGF,

in which agonists are identified as those peptides that inhibit the binding but mimic the cellular effects of Flk-1.

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24. The method according to Claims 22 or 23 in which the Flk-1 protein is genetically engineered.

5 25. A method of modulating the endogenous enzymatic activity of the tyrosine kinase Flk-1 receptor in a mammal comprising administering to the mammal an effective amount of a ligand to the Flk-1 receptor protein to modulate the enzymatic activity.

10 26. The method of Claim 25 in which the ligand to the Flk-1 receptor is VEGF.

15 27. The method of Claim 25 in which the ligand to the Flk-1 receptor is a VEGF agonist.

28. The method of Claim 25 in which the ligand to the Flk-1 receptor is an antagonist of VEGF.

20 29. The antagonist of Claim 28 that is a monoclonal antibody which immunospecifically binds to an epitope of Flk-1.

30. The antagonist of Claim 28 that is a soluble Flk-1 receptor.

25 31. The method of Claim 25 in which the enzymatic activity of the receptor protein is increased.

30 32. The method of Claim 25 in which the enzymatic activity of the receptor protein is decreased.

33. The method of Claim 31 in which the ligand stimulates endothelial cell proliferation.

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-66-

34. The method of Claim 32 in which the ligand inhibits endothelial cell proliferation.

35. The method of Claim 32 in which the ligand 5 inhibits angiogenesis.

36. A recombinant vector containing a nucleotide sequence that encodes a truncated Flk-1 which has dominant-negative activity which inhibits the cellular 10 effects of VEGF binding.

37. The recombinant vector of claim 36 containing a nucleotide sequence encoding amino acids 1 through 806 of Flk-1.

15 38. The recombinant vector of claim 36 in which the vector is a retrovirus vector.

39. The recombinant vector of claim 38 containing 20 a nucleotide sequence encoding amino acids 1 through 806 of Flk-1.

40. An engineered cell line that contains the recombinant DNA vector of Claim 36 and expresses 25 truncated Flk-1.

41. An engineered cell line that contains the recombinant vector of Claim 38 or 39 and produces 30 infectious retrovirus particles expressing truncated Flk-1.

42. An isolated recombinant truncated Flk-1 receptor protein which has dominant-negative activity which inhibits the cellular effects of VEGF binding.

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-67-

43. A method of modulating the cellular effects of VEGF in a mammal comprising administrating to the mammal an effective amount of truncated Flk-1 receptor protein which inhibits the cellular effects of VEGF binding.

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| | | | |
|-------|-----|-------------------------------------------------------------|-----|
| FLK-1 | 866 | ILIHIGHHLNWNLLGACTTKPGPLMWIVFSKEFGNLSYLRGKRNRFVPYKSKGARFRQ | |
| KDR | | | C—D |
| TKR-C | | | C |
| FLK-1 | 926 | GKQYVGEISVOLKRLDSITSSQSSASSGFVEEKSLSDVEEEASEEELYKDFLTLEHLIC | |
| KDR | | | AIP |
| TKR-C | | | P-D |
| FLK-1 | 986 | YSEQVAKGAEFLASRKCIHRLAARNILLSEKWWKICDFGLARDIYKQDYYRKGDARL | |
| KDR | | | |
| TKR-C | | | |

FIG. 1

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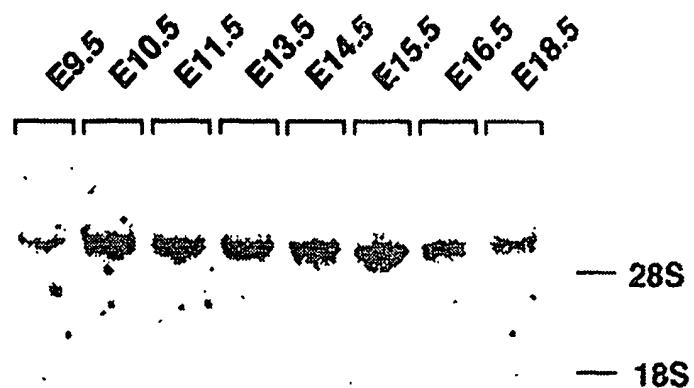


FIG. 2A

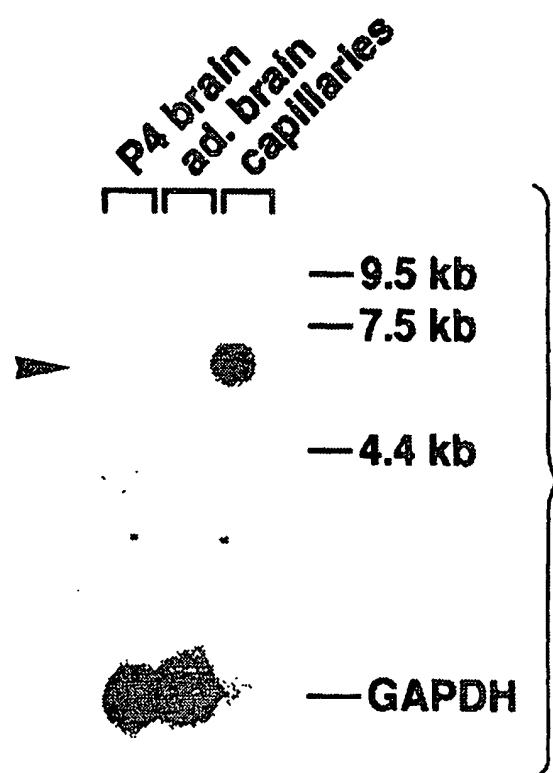


FIG. 2B

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Ms



FIG. 3A

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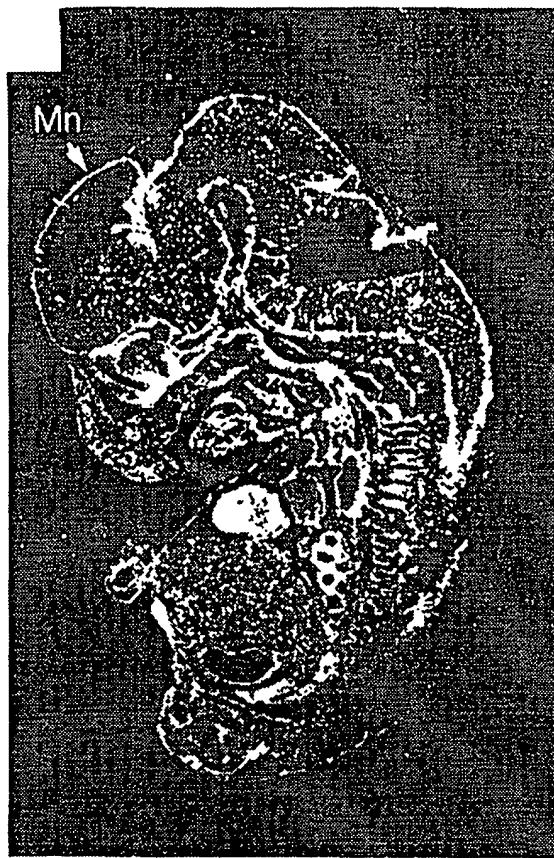


FIG. 3B

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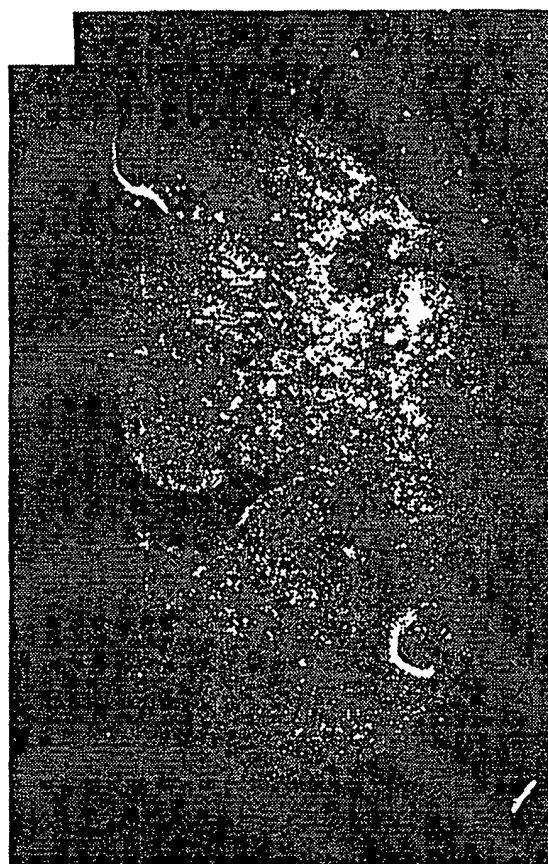


FIG. 3C

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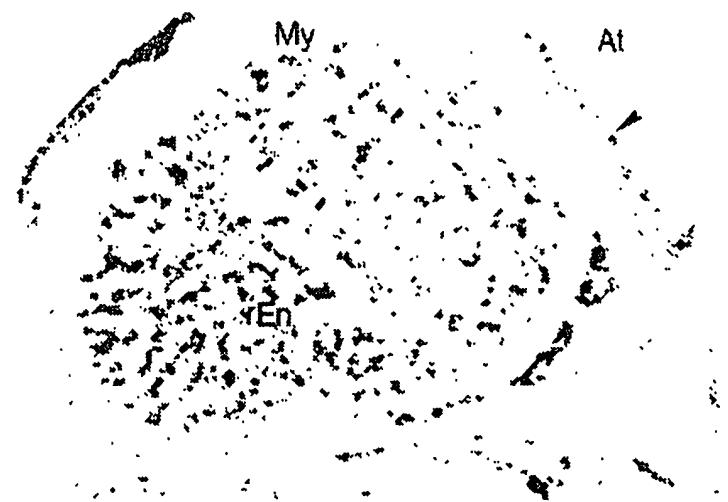


FIG. 4A



FIG. 4B

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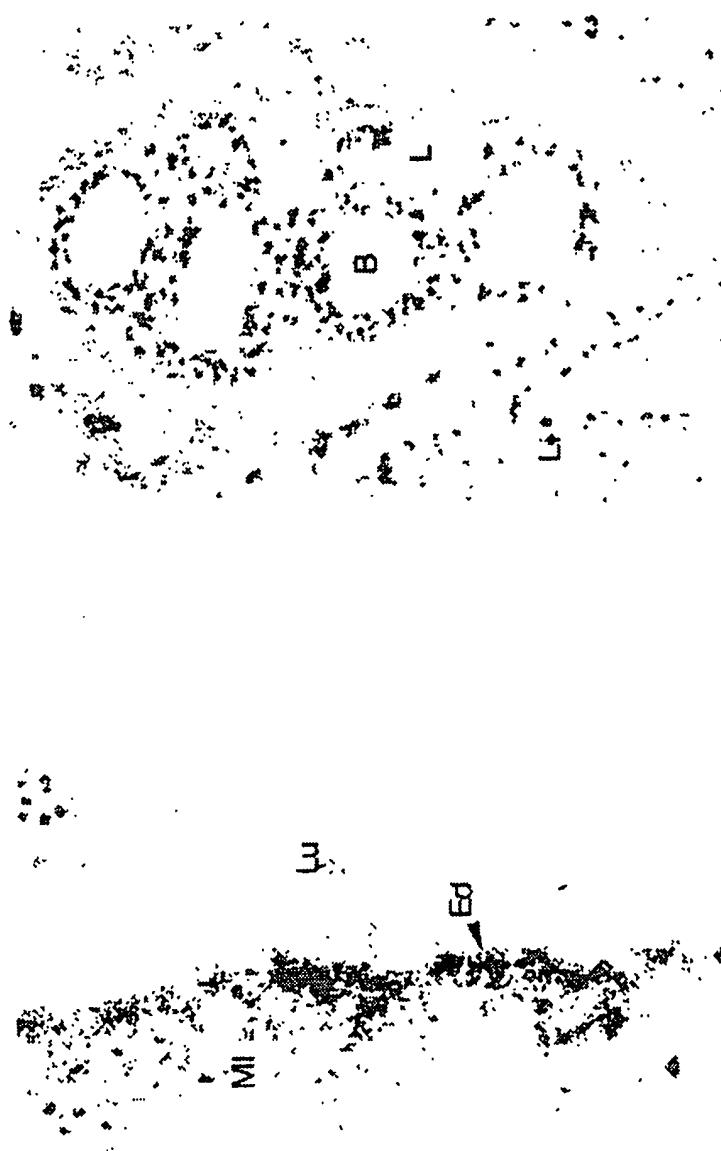


FIG. 4C

FIG. 4D

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FIG. 4E

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FIG. 5A

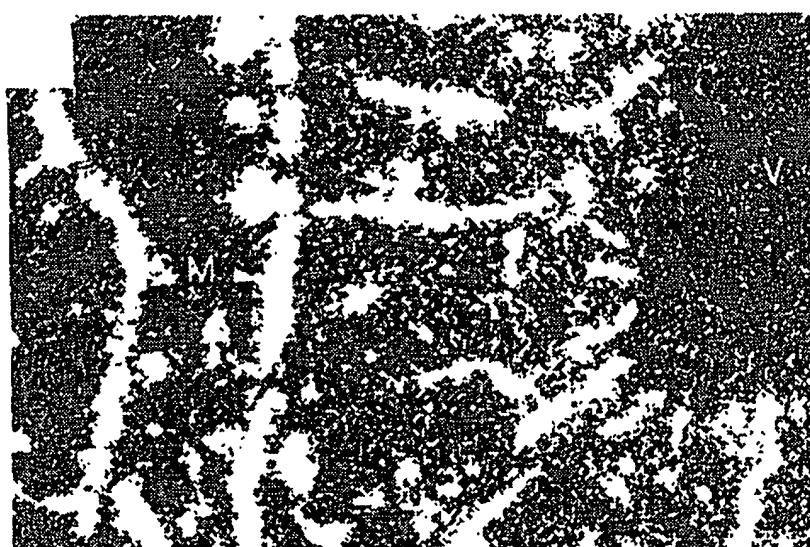


FIG. 5B

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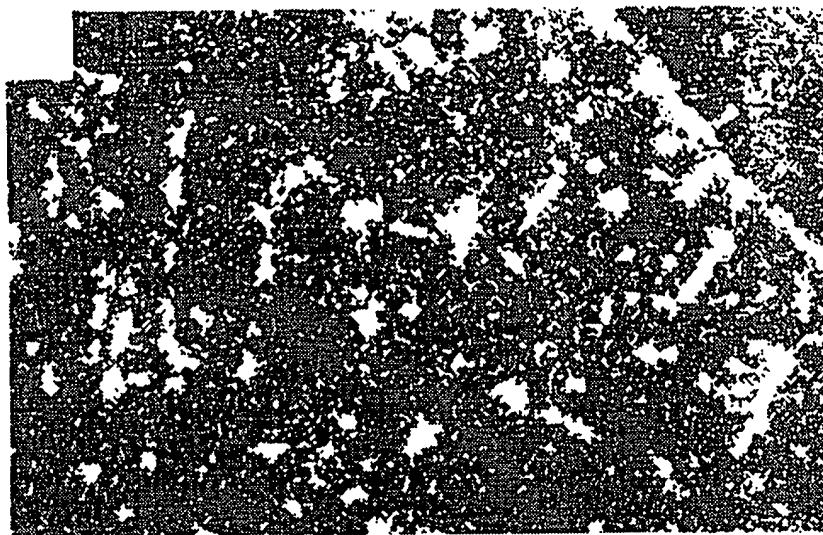


FIG. 5C

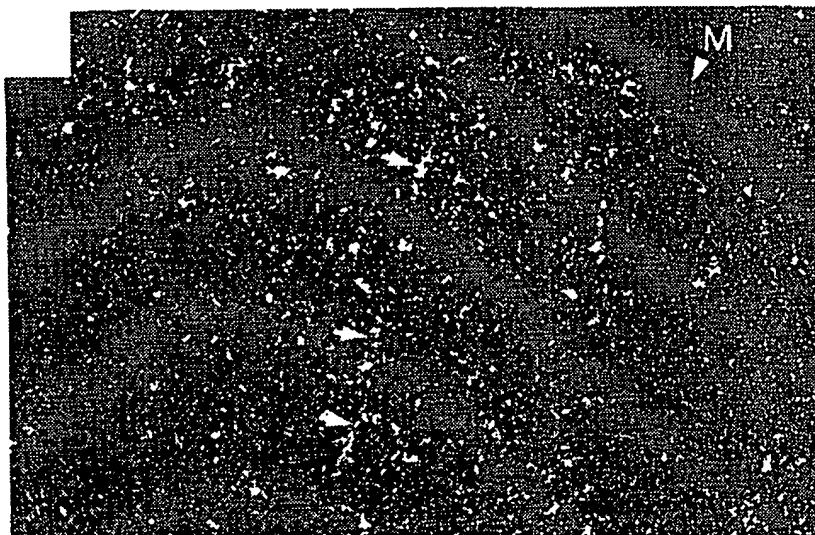


FIG. 5D

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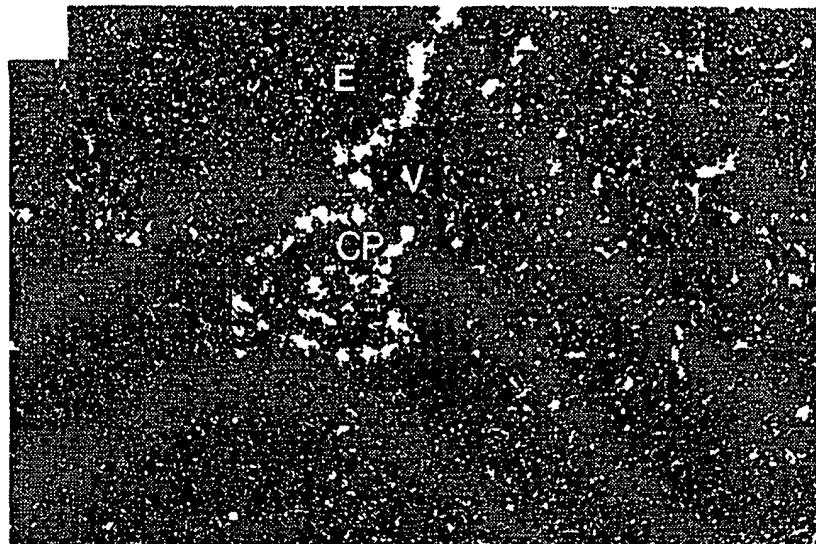


FIG. 6A



FIG. 6B

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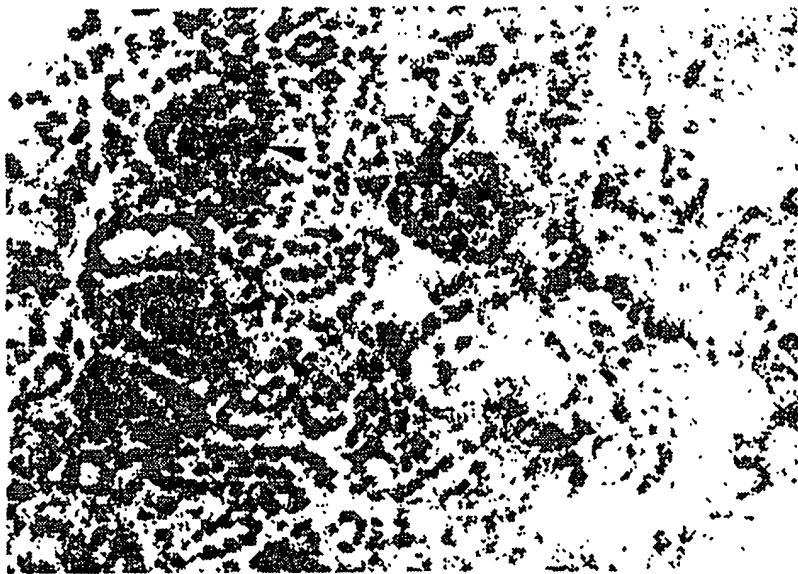


FIG. 7A

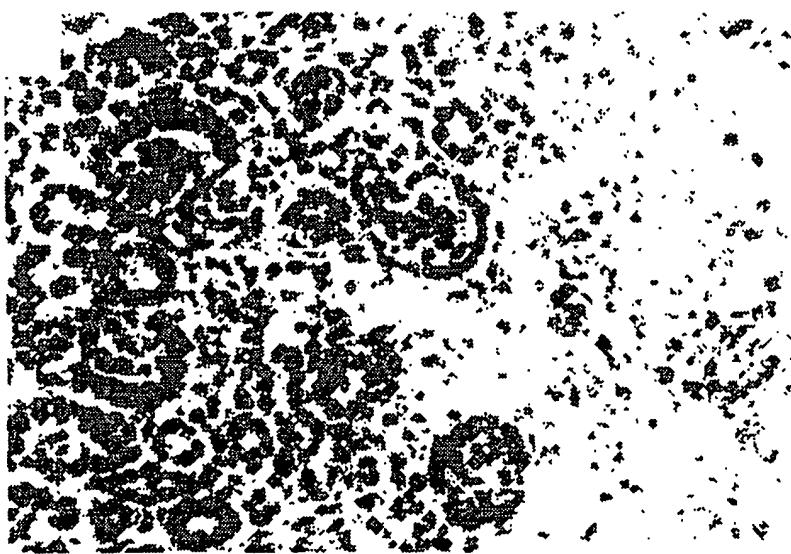


FIG. 7B

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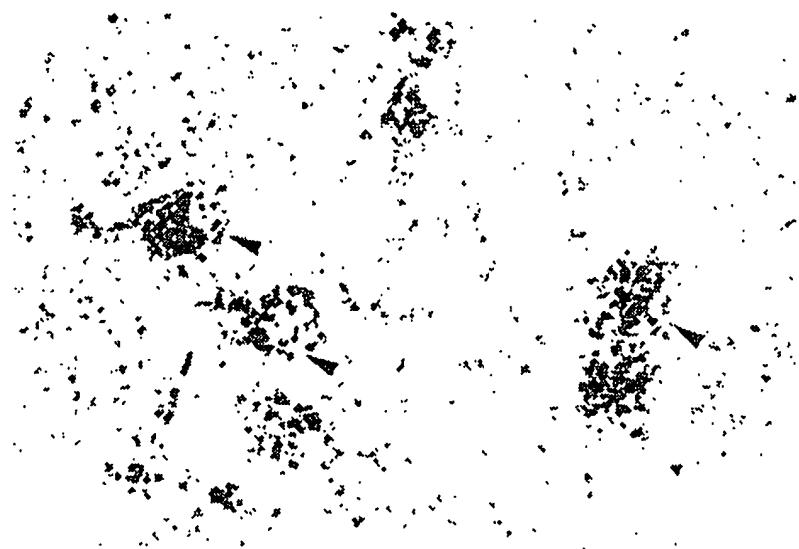


FIG. 7C

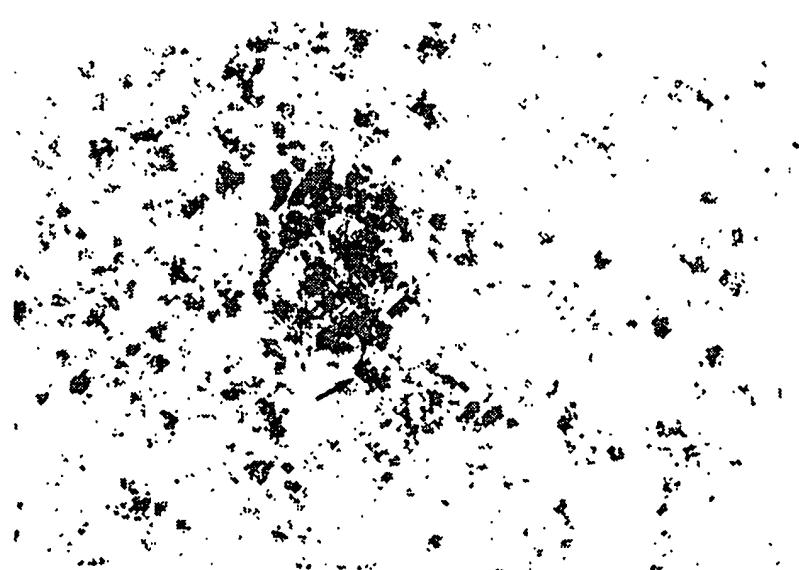


FIG. 7D

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FIG. 8B

FIG. 8A

D

2BV

NF

Y

M

15/26



FIG. 8D

FIG. 8C

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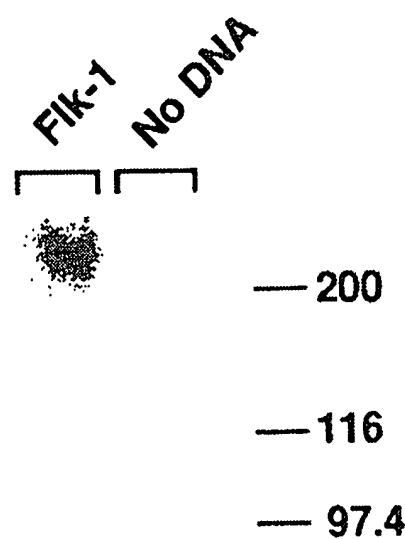


FIG. 9A

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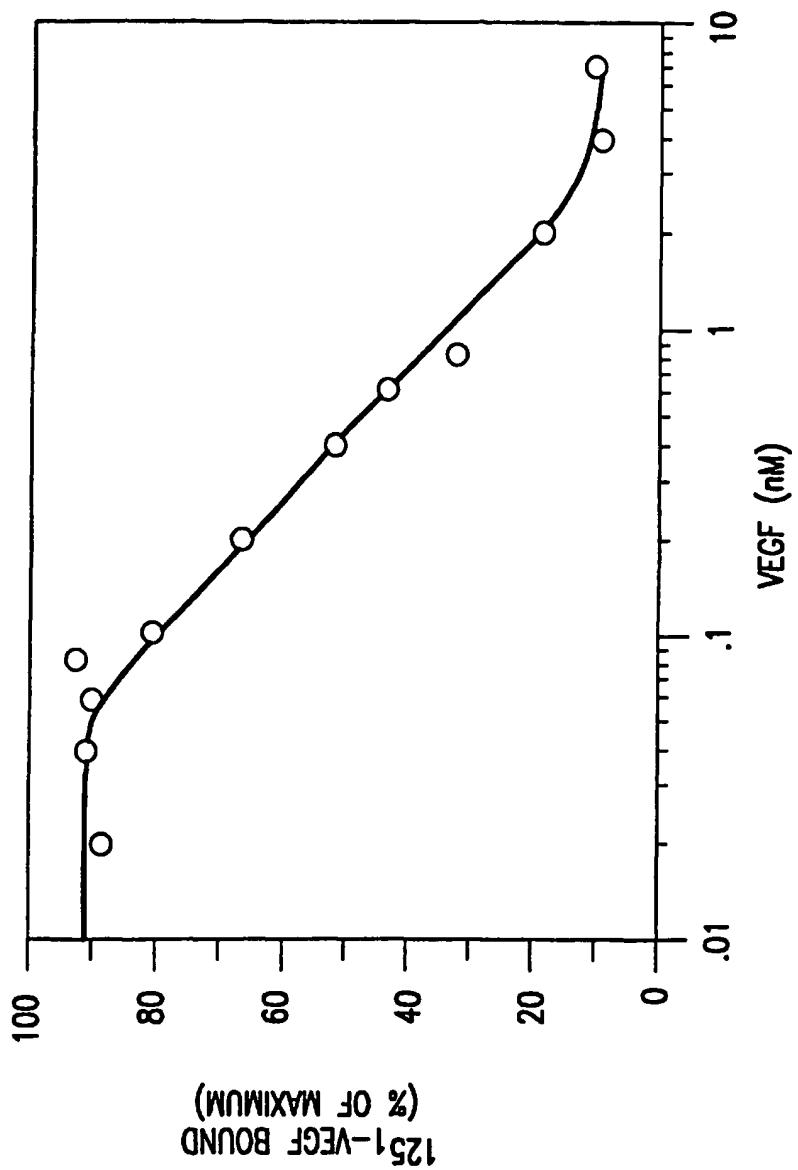


FIG. 9B

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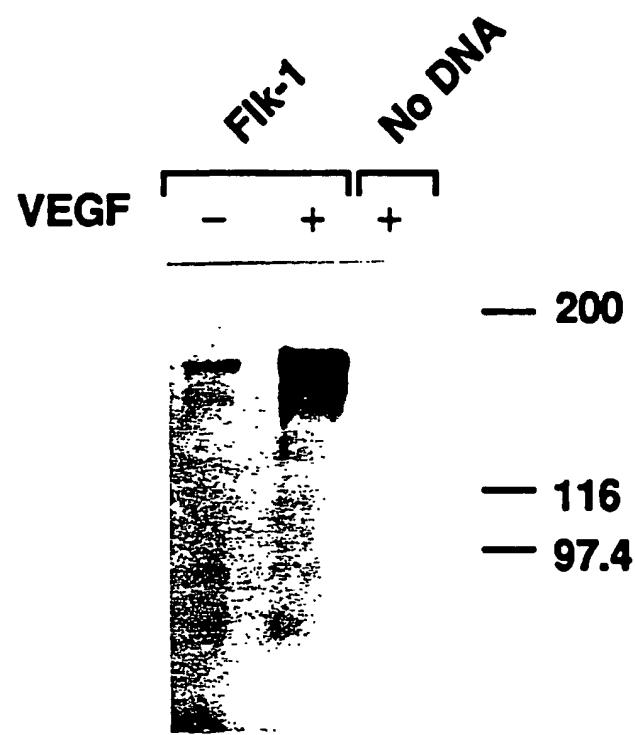


FIG. 10

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1 TATAACGGCAATTGGTACGGGACCCCTCCAGTCCACCGTATCCATAAGCTGATATCCAATTGGGAGACTGTCTCCCCCAGC 90
 91 CGGATAACCTGGTACGGGATTCGGGAGACCGCTGAGACGGGGGCTGGACCCAGGGGGGGGGCTCTCCCCCTCTT 160
 181 CGGCTGGGGGGCCATACCCCTCTGACTCTTGGGGCCAGGGACGGAGAACGACTCTGCGCTGAGAAACTGGCTCTGCGCA 270
 MESKALLAVALWFCVHTRAASVGLT 25
 271 CGGGGAGGTGACCGATGGAGACCAAGGGCTGCTACCTGCGCTCTGCGCTGGAGACCCAGGGCTCTGCGCTTGA 360
 26 GDFLHPPKLS T Q K D I L T I L A N T T L Q I T C R C 55
 361 CGGGATTTCTCCATCCCCAACCTCACCAACAGAACATACTGACAATTGGCAAATACAACCTTCAGATTACTGGCACCGA 450
 56 Q R D L D K L M P N A Q R D S E H R V L V T E C G G G D S I 85
 451 CAGGGGACCTGGACTGGCTTGGCCAATCCTCACCGTATTCTGAGGAAGGTATTGGTACTGAATGGGGCTGGTACACTAC 540
 86 F C K T L T I P R V V G N D T G A Y K C S Y R D V D I A S T 115
 541 TTCTGCAAAACACTCACCATCCACGGCTCTGGAAATGATACTCGACCTACAAGTCTCTACCGAACCTCCACATACCTCCACT 630
 116 V Y V Y V R D Y R S P F I A S V S D Q H G I V Y I T E N K N 145
 631 CTTTATCTCTATCTGAGATTACAGATCACCACTACCCCTCTGACTGACCCATGGCATCTGACATCACCGAGAACAAAC 720
 146 K T V V I P C H G S I S N L N V S L C A R Y P E K R F V P D 175
 721 AAAACTCTCTGATCCCCCTGGAGGGCTGGATTCTAAACCTCAATGTCCTCTTGGCTAGGTATCCAGAAAACAGATTGTTCCCGAT 810
 176 G N R I S K D S H I G F T L P S Y M I S Y A G M V F C E A K 205
 811 CGAACAGAAATTCTGGGACACGGAGATGGCTTACTCTCCAGTACATGATCACGCTATGGGGATCTCTGACCCAAAC 900
 206 I N D K T Y Q S I M Y I V V V V G Y R I Y D V I L S P P H H 235
 901 ATCAATGATGAAACCTATCAGTCTATCATGATACTCTCTGCTCTAGGATATGGATTATGATGTCATCTGAGCCCCCCCCATGAA 990
 236 I K L S A G K K L V L N C T A R T E L N V G L D F T M H S P 265
 991 ATTGACCTATCTGGGAGAAAACCTGCTTAAATTGACGGAGAACAGACCTCAATGTCGGCTTGATTCACTGGCAACTCTGCA 1080
 266 P S K S H H K K I V N R D V K P F P G T V A K M F L S T L T 295
 1081 CCTTCAAACTCTCATCATAGAACATTGTAACACGGATGAAACCCCTCTGGACTCTGGCAAGATGTTTGACCACTTGACA 1170
 296 I E S V T K S D Q G E Y T C V A S S G R M I K R N R T F V R 325
 1171 ATAGAAACTGACCAAGACTGACCAAGGGAAATACACCTGTCAGGCTCCACTGGACGGATCATCAAGAGAAATAGAACATTGTCGA 1260

FIG.11A

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326 V H T K P F I A F G S G M K S L V E A T V G S Q V R I P V K 355
 1261 G T T C A C A A A G C C T T T A T T G C T T T C G G T A C T G C C A T G A A A T C T T G G T G G A A G C C A C A G T G C C C A C T C A A C T C C C A A T C C C T G A A G 1350
 356 Y L S Y P A P D I K N Y R N G R P I E S N Y T M I V G D K L 385
 1351 T A T C T C A G T T A C C C A G T C T G A T A T C A A A T C G T A C A G A A A T G C A A G C C C A T T G A G T C C A A C T A C A C A T G A T T G C C C A T G A A C T C 1440
 386 T I M K V T K R D A Q N Y T V I L T N P I S N E K Q S H M V 415
 1441 A C C A T C A T C G A A G T G A C T G A A A G A G A T G C A C G A A A C T A C A C C G T C A T C C T C A C C A A C C C A T T C A A T G G A G A A A C A G A C C C A C A T G C T C 1530
 416 S L V V K V P P Q I G E K A L I S P M D S Y Q Y G T M Q Y L 445
 1531 T C T C T G G T T C T G A A T G T C C C A C C C C A G A T C G G T G A A A G C C T T G A T C T G C C T A T G G A T T C C T A C C A G T A T G G A C C A T G C A G A C A T T G 1620
 446 T C T V Y A N P P L H H I Q N Y N Q L E E A C S Y R P G Q T 475
 1621 A C A T G C C A C A G T C T A C G C C A A C C C T C C C T G C A C C A C A T C C A C T G G T A C T G G C A C C T A G A A C A A G C C T C C T C A C A G A C C C C C A A A C A 1710
 476 S P Y A C K E K R H V E D F Q G G N K I E V T K N Q Y A L I 505
 1711 A G C C C T A T G C T G T A A G A A T G G A G A C A C G T G G A C C G T T C C A C G G G G G A A C A A G A T G A A G T C A C C A A A C C A A T A T G C C C T G A T T 1800
 506 K G K N K T V S T L V I Q A A N V S A L Y K C E A I N K A C 535
 1801 G A A C G A A A A A C A A A A C T G T A A C T T A C C C T G G T C A T C C A A G C T G C C A A C C T G T C A G C C T T G T A C A A A T C T G A C C C A T C A A C A A A G C C G A 1890
 536 R G E R V I S F H V I R G P E I T V Q P A A Q P T E Q E S V 565
 1891 C G A G G A G A G G G T C A T C T C C T T C C A T G T G A T C A G G G T C C T G A A A T T A C T G T G C C A A C C T G C T G C C C A C C A A C T G A G C C G A G A C T G T C 1980
 566 S L L C T A D R N T F E N L T N Y K L G S Q A T S V H N G E 595
 1891 T C C C T G T G C A C T G C A G A C A C A A A T C G T T G A A C C T C A C C T G C C T C A C A G C C A A C A T G C T C A C A T G G G C G A A 2070
 596 S L T P V C K N L D A L N K L M G T M F S N S T N D I L I V 625
 2071 T C A C T C A C A C C A G T T G C A A C A T T G C A T G C T C T T G G A A C T G A A T G C C A C C A T G T T T C T A A C A C C A A A T C A C A T T G C T G T C 2160
 626 A F Q N A S L Q D Q G D Y V C S A Q D K K T K K R H C L V K 655
 2161 G C A T T C A G A A T G C C T C T G C A G G A C C A A G G C A C T A T G T T C C T C T G C T C A A G A T A A G A A G C C A A G A A A G C A T T G C C T G T C A A A 2250
 656 Q L I I L K R M A P H I T G N L S N Q T T T I Q E T I H V T 685
 2251 C A G C T C A T C C T A G A G C C A T G C C A C C C A T G A T C A C C G G A A A T C T G G A G A T C A G A C A C A A C C A T T G C C G A G A C C A T G A A G T G A C T 2340
 686 C P A S C N P T P N I T K F K D N E T L V E D S G I V L R D 715
 2341 T G C C C A G C A T C T G G A A A T C C T A C C C C A C A C A T T A C A T G C T C A A A G A C A A C G A G A C C C T G G T A G A A G A T T C A G G C A T T G T A C T G A G A G A T 2430
 716 G N R N L T I R R V R K E D G G L Y T C Q A C N V L G C A R 745
 2431 C G G A A C C G A A C C T G A C T A T C C C A G G G T G A C C A A G G G A T G C A G G C C T C A C A C C T G C C A G G C C T G C A A T G T C C T T G C C T G T C C A A G A 2520

FIG.11B

SUBSTITUTE SHEET

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716 A E T L F I I E G A Q H K I N L E V I I I L V G T A V I A M F 775
 2521 C C G G A G C C T C T C A T A A T G A A C G T G C C C A G G A A A G C C A A C T T C C A A C T C A T T A C C T C T C G C A C T G C A C T G A T T C C A T G T C 2610

776 F M L L L V I V L R T V K R A N H G K L K T G Y L S I V M D 805
 2611 T T C T G G C T C C T T C T C A T T C T C A C G G A C C T T A A C C G G G C C A A T G A A G C C G A A C T G A A G A C A G G C T A C T T C T C A T T C T C A T G C A T 2700

806 F D K L P L D H R C K E L P Y D A S K N E F P R D R L K L G 835
 2701 C C A C A T G A A T T G C C C T T G C A T C A G C C T T G C C T T A T G A T G C C A G C A A T T C C C A C C G A C C G A C C G C T G A A A C T A G G A 2790

836 K F L G R G A F G Q V I E A D A F G I D K T A T C K T V A V 865
 2791 A A A C C T C T T G G C C C C C G T C C C T T C C C C A A G T G A T T G A C C C A G A C C T T T G G A A T T G A A A G A C A C C A C T T C C A A A C A G T A C C C G T C 2880

866 K M L K E G A T H S E H R A L M S K L K I L I H I G H H L M 895
 2881 A A C A T G T C A A A C C G A C C A A C A C A C G C A C C A T G C A C C C T C A T G T C A A C T C A A G A T C C T C A T C C A C A T T G G T C A C C A T C T C A A T 2970

896 V V N L L G A C T K P G G P L M V I V E F C K F G N L S T Y 925
 2971 G T C G T G A A C C T C C T A G G G C C T T G C A C C A A C C C G A G G C C C T C T C A T G C T G A T T G T G G A A T T C T C C A A C T T T G G A A A C C T A T C A A C T T A C 3060

926 L E G K R N E F V P Y K S K G A R F R Q G K D Y V G K L S V 955
 3061 T T A C C G G C A C A G A A A T C A A T T T C C C T A T A C C C A A A G G C C A C C T T C G C C A G G C C A A G G A C T A C C T T C C C A G C C T C T C C T G 3150

956 D L K R R L D S I T S S Q S S A S S G F V K H K S L S D V E 985
 3151 G A T C T G A A A A G C C C T T G A C A C C A T C A C C A C C C A G C C T C G C C A C C T C A G G C T T T G T G A C C G A A A T C C C T C A G T G A T G A G 3240

986 K K K A S K K L Y K D F L T L K H L I C Y S F Q V A K G M E 1015
 3241 G A A G A A G A C T T C T G A A G A C T G T A C A A C G A C T T C T G A C C T T G G A C C A T C T C A T C T G T T A C A C C T T C C A A C T G G C T A A G G C A T G G A G 3330

1016 F L A S R K C I H R D L A A R N I L L S E K N V V K I C D F 1045
 3331 T T C T T G G C A T C A A G A C T G T A T C C A C A C G A C C T T G G C A G C A C C A A A C T T C T C T A T C C G A A G A A T G T G G T T A A G A T C T G T G A C T T C 3420

1046 G L A R D I Y K D P D Y V R K G D A R L P L K K M A P E T I 1075
 3421 G G C T T G G C C C G G A C A T T T A T A A G A C C C G A T T A G T C A G A A A A G G A G A T G C C C A C T C C C T T G A A G T G G A T G C C C C G G A A A C C A T T 3510

1076 F D R V Y T I Q S D V N S F G V L L N E I F S L G A S P Y P 1105
 3511 T T T G A C A G A C T A T A C A A T T C A G A C C G A T G T G T C T T T C C G T G T G C T C T G G A A A T T T T C C T T A C G T G C T C C C C A T A C C C T 3600

1106 G V K I D E E F C R R L K E G T R M R A P D Y T T P E M Y Q 1135
 3601 G C C G T C A A G A T T C A G A A G A T T T T G A C C A T T C A G A A C C A A C T A G A A T G C C G G C T C T G A C T A C A C T A C C C C A G A A A T G T A C C A G 3690

FIG.11C

SUBSTITUTE SHEET

1135 T N L D C N H E D P N Q R P S F S E L V E H L G N L L Q A N 1165
 3691 ACCATGCTGACTGCTGGATGAGGACCCCCAACCAAGAGACCTCTGTTTCAAGACTTGCTGACCATTTGGAAACCTCTGCAAGCAAAT 3780

1166 A Q Q D G K D Y I V L P M S E T L S M K E D S G L S L P T S 1195
 3781 GCGCGCCACCATCCCCAAACATATTTCTCTCCAAATCTCACAGACACTCACCATGGAGAGGATTCTGGACTCTCCCTGCCAACCTCA 3870

1196 P V S C M E E E H V C D P K Y H Y D N T A G I S H Y L Q N S 1225
 3871 CCTGTTCTGTATGCCAACAGAACGAAAGTGTGCGACCCCCAATTCCATTATGACAACACAGCAGGAATCAGTCATTATCTCAGAACAGT 3960

1226 K R K S R P V S V K T F H D I P L E E P E V K V I P D D S Q 1255
 3961 AACGGAAACAGCCCCCAGTCACTGTAACATTGAGATATCCATTGGACCAACAGCAGGAATCAGTCATTATCTCAGAACAGT 4050

1286 T D S G M V L A S E E L K T L E D R N K L S P S F G G M M P 1285
 4051 ACAGACAGTGGATGCTCTGCATCAGAACGCTGAAACTCTGGAAGACAGAACAAATTATCTCCATCTTCTGGAAATGATGCCAGATGACAGCCAG 4140

1286 S J S R E S V A S E G S B Q T S G T Q S G T G S D D T D T T 1315
 4141 ACTAAACACGGACTCTCTGCCCTCGAACCCCTCAACAGACCACTGCCCTACAGCTCGGTATCACATCAGATGACACAGAACCCACC 4230

1316 V Y S S D E A G L L K M V D A A V H A D S G T T L Q L T S C 1345
 4231 GTCTACTCCAGGACGGAGGAGCTTTAAACATGGATGCTGAGTTACGGCTACTCAGGACACTGGGACCCACTGGAGCTCACCTCGT 4320

1346 L N G S G P V P A P P P T P G N H E R G A A + 1367
 4321 TAAATGGAAGTGGCTCTGCTCCCGCTCGCCCCCAACTCTGAAATCAGAGAGAGCTGCTTAGATTTCAGTGTGTTCTTC 4410

4411 CACCAACGGAACTAGCCACATTGATTTCATTTGGACGGAGGACCTCAGACTGCAAGGACCTGCTCTCAGGGCATTTCCAGACAA 4500

4501 GATGCCATGACCAAGATGTTGACTCTACTCTCTTCCATTCTTAAACCTCTATATAATGCTCCCTGCTGCTACTAC 4590

4591 CAGTTAACCAAAAGACTTCAAAACAGTGGACTCTGCTCCAAAGACTGCCAACCCACCTCTGAAACTGGATGCAATGCCAATG 4680

4681 CTTCGCTGAGATGGTGGACTGGAGGAAGGAAGGCCAAGTGGCTGGAGGCTGGCTGGCTGGAGATCCATTGCTGGCTGGT 4770

4771 TAAGTGTGGATGTGGACTGGAGGAAGGAAGGCCAAGTGGCTGGAGGCTGGCTGGAGATCCATTGCTGGCTGGTGG 4860

4861 ACCTGGCTTGCCCTGTCAGGAAACCAAACGGGGGGGGAGGTTCGTTGGACCTTGGCTGGCTCTCACAGTGGCTTACAG 4950

4951 CGCACTTCCCTGTCGCCCTTCTACTCTTAATGAGACTCTTCCCTGACTCTAACGTCCTCTGGCTGGCCCCAGGAAGGAATGATG 5040

5041 CACCTGGCTCTCTCATCTCAGGCTGTCCTTAATTCAAAACACAAAGAGAGGAACTGGCTCCACAGGCTCTGACGGGGCGAA 5130

5131 GAATTGAGAAACAGAACAGAAACTCACCGTTCTGCTGGTGGAGACCCACGTGGGGCTGGTGGCACGCTGACGGTTCTCTGCAA 5220

5221 GTGGCGTAAAGCTCACCTGGCTCTCTCTCTATCTCCACTCTGTCAGGCCCCAACCTCTCACTATTTCAGCTTGTGGCTTCT 5310

5311 GATGCCAGAAAATCTTAATTGGTGGCTTCAGATAATCACTACCCAGATTGAAATTACTTTAGGGAGCTATGATAAC 5400

5401 ATCTACTGATCTTAACTATAAAACTATGCTACTGGTTCTGCTCTGCTTATGTT 5470

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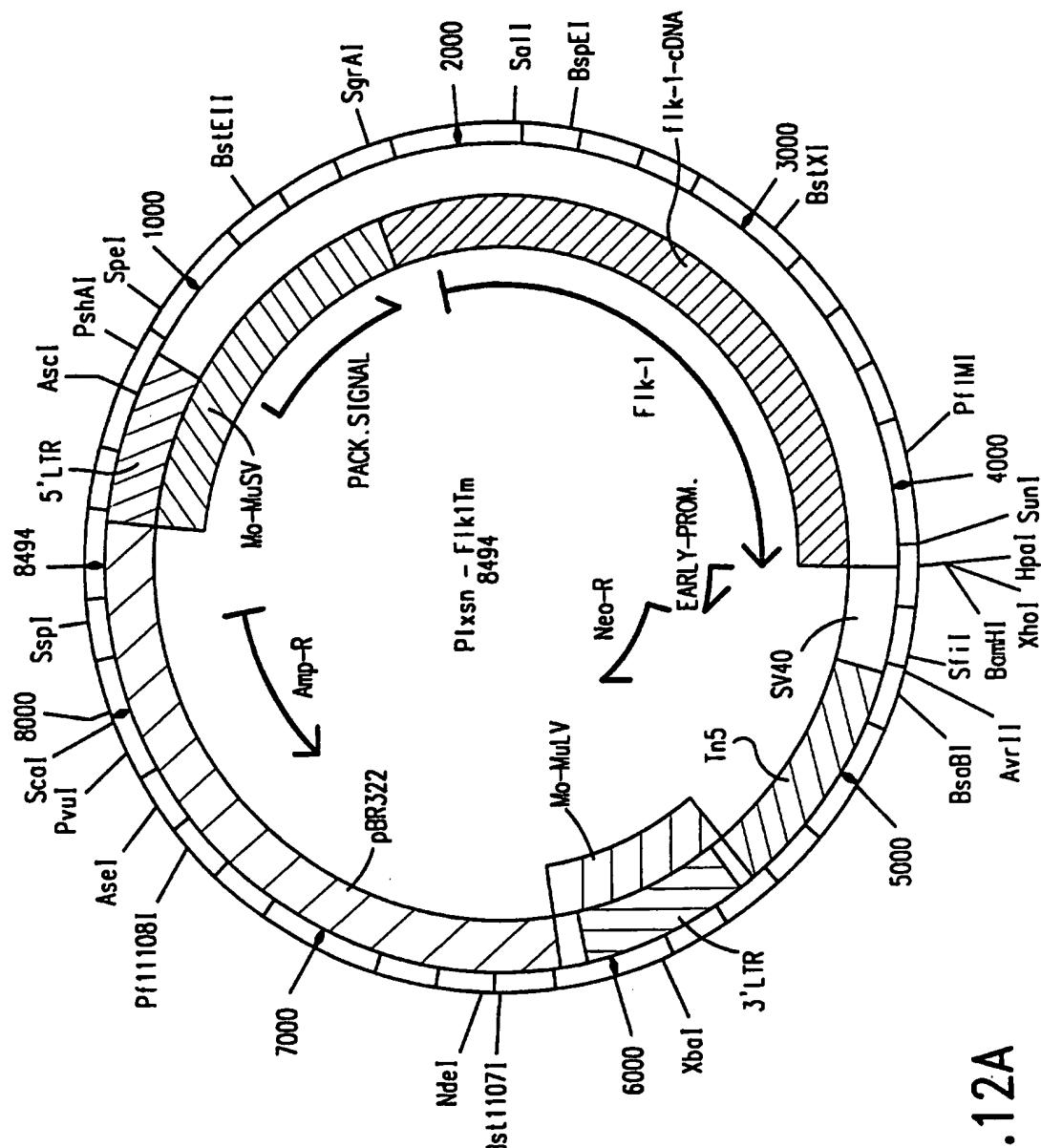


FIG.12A

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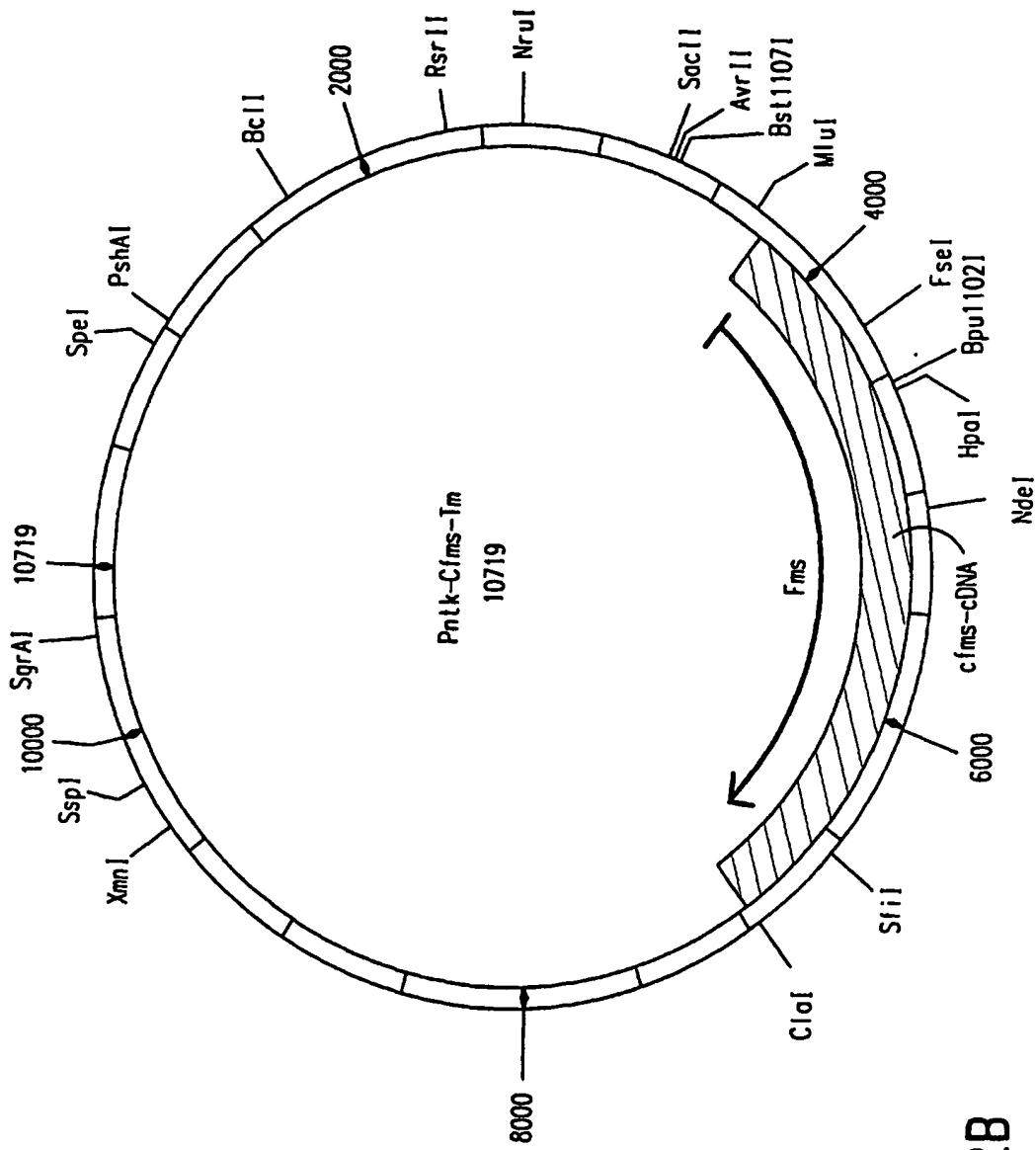
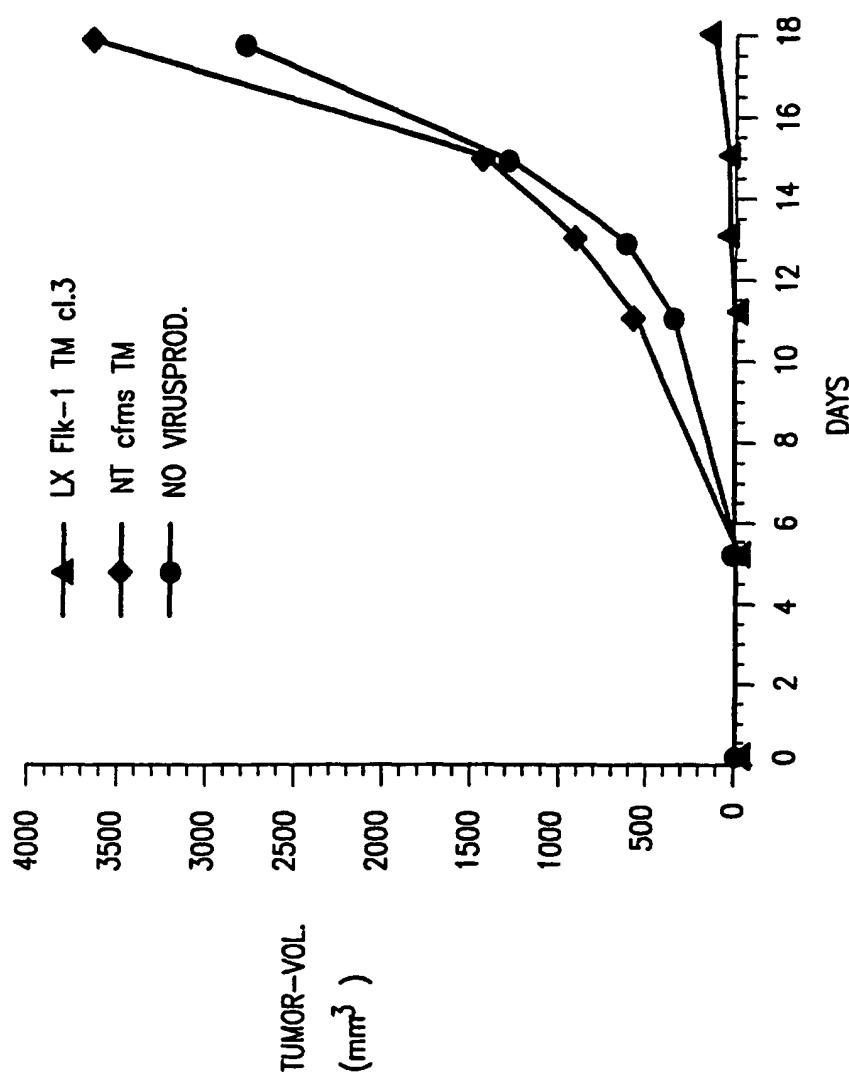


FIG. 12B

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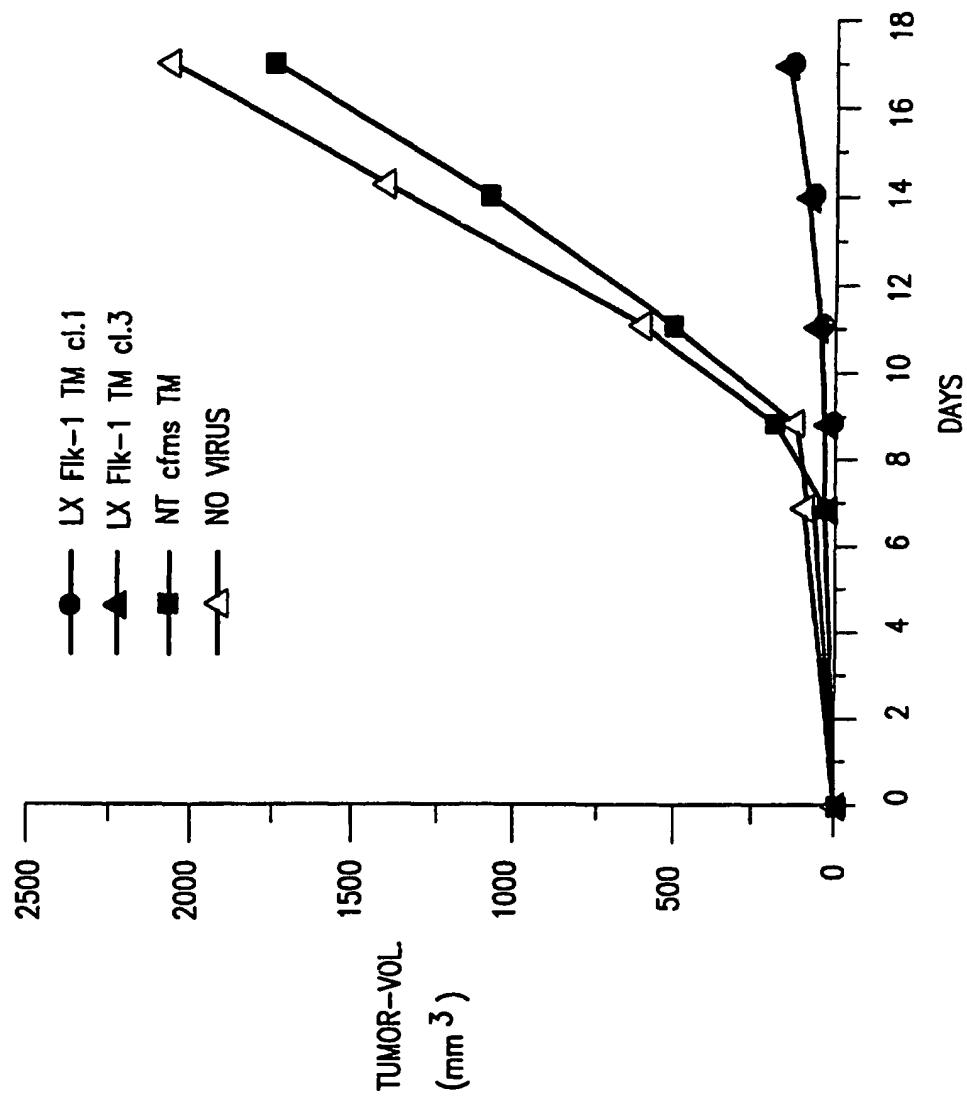


FIG. 14

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